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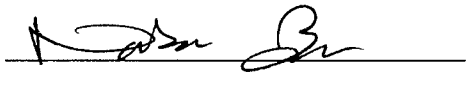

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13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*

Purpose & scope: The discovery of p73, a gene that in experimental conditions behaves like p53, requires us to determine what role it plays in breast cancer, whether a crosstalk exists between p73 and p53 actions and to delineate the differences and similarities between these two genes concerning their biological role and signaling pathways. Our understanding of p53's role in breast cancer has been made hazier again by the advent of p73's discovery. An additional challenge derives from the fact that the TP73 gene in principle can produce two diametrically opposed classes of protein products:full length forms and N-terminally truncated forms (that are missing the transactivation domain). Therefore, certain p73 isoforms could be dominant negative over p53 in heterotypic interactions. Conversely, it has already been shown that p73's transactivation and apoptotic function is inhibited by tumor-derived p53 mutants. This opens the possibility that the phenotype of mutant p53 tumor cells might in fact be due to an interference with normal p73 function. This scenario might explain why only 30 % of breast cancers have mutated their p53 gene. In this case, dominant negative p73 isoforms, when deregulated in breast cancer, could interfere with p53 and p73-mediated growth suppression. We had previously reported that about 40% of breast cancers overexpress p73, indicating its role in breast cancer tumorigenesis. A better insight into p73's function will add greatly to our understanding of its role in this disease. Major findings: A) We showed that certain relevant oncogenes signal to p73 *in vivo*. Endogenous full length (TA) p73 α and β proteins are upregulated and transcriptionally activated in p53-deficient tumor cells in response to deregulated oncogenes E2F1, c-MYC and E1A. Moreover, in the absence of p53, E2F1, c-MYC and E1A enlist p73 to induce apoptosis in tumor cells. B) Δ Functional studies indicate that ΔNp73 acts as a dominant negative inhibitor of both wild-type p53 and transactivation-competent TA-p73.

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Introduction:

This grant was divided into two unrelated parts due to an approved change of scope for years 2-3. The first part deals with the risk of ATM heterozygosity for sporadic breast cancer, while the second deals with the role of the p53 homolog p73 in breast cancer.

Body:

Part I: Risk for Sporadic Breast Cancer in Ataxia Telangiectasia Heterozygotes

Scope: The scope was to assess whether heterozygosity for the ATM gene, due to a loss of function mutation in one of the 2 alleles and found in about 1% of the general population, confers a significant increase in breast cancer risk for women with sporadic breast cancer (without a family history of breast cancer). This is called the AT - carrier risk hypothesis for sporadic breast cancer.

The goals for the first 12 months were:

Aim I Genetic analysis of ATM in clinical samples.

IA) LOH mapping at 11q23.1 in sporadic breast carcinomas using intragenic and ATM flanking microsatellite markers (months 1-18).

Time Table

l LOH mapping at 11q23.1 in breast carcinoma/normal tissue matched pairs using intragenic and ATM flanking microsatellite markers (months 1-18)

l LOH mapping at 11q23.1 in DNA from normal controls using intragenic and ATM flanking microsatellite markers (months 1-18)

Reportable results in LOH mapping:
Using 6 polymorphic microsatellite markers in and around the ATM locus, we completed LOH analysis on 16 matched breast cancer/normal pairs with the following results:

D11S2179 (intragenic ATM):	4 of 16 (25%)
NS22 (intragenic ATM):	3 of 16 (19%)
D11S1787 (centromeric):	4 of 16 (25%)
D11S1778 (telomeric):	6 of 16 (38%)
D11S1294 (telomeric):	6 of 16 (38%)
D11S1818 (telomeric):	4 of 16 (25%)

Interpretation: Our results on frequencies of the ATM and flanking loci in breast cancer is similar to the ones reported in the literature.

In summary, our LOH results only confirmed the frequency data was already in the literature. Furthermore, the new mutational studies that appeared in the meantime did not show a significant mutational rate of the ATM gene. The latter is a strong but not absolute argument against a true suppressor role of the ATM gene in breast cancer. Rather than simply to continue LOH analysis on the originally planned 145 total cases, we decided to address the question from a different angle. We asked whether the expression status of ATM differed in breast cancers and breast cancer cell lines compared to normal breast tissue. If ATM has a suppressor role in breast cancer, a loss of wild type ATM expression rather than mutational inactivation could be expected.

With this rationale, we undertook a comprehensive ATM expression analysis using quantitative RT-PCR on 89 randomly selected breast cancer samples (from 3 different institutions), 7 breast cancer lines and 29 normal breast samples. Of these, 11 were matched normal/cancer pairs. Our working hypothesis was to find a decreased expression in cancer compared to normal breast tissue.

Results of ATM expression in breast cancer and normal breast tissues. Using a competitive semiquantitative RT-PCR approach, we determined relative ATM expression levels on 89 breast cancers and compared them to 29 normal breast samples. Eleven of these constituted matched tumor/cancer pairs. ATM and b2M transcripts were detectable in all breast tissues and the 7 breast cancer cell lines that we analyzed. While the expression of b2M was similar in all samples, ATM expression levels varied widely. Moreover, breast cancer tissues did not show a deficiency in ATM expression. In fact, cancers expressed mildly higher (1.5-fold) levels of ATM transcripts than normal breast tissues. However, due to the large variance in breast cancers and the relatively small difference between the geometric means of cancer versus normal tissue, the power to detect significant differences between the two groups was very low. The geometric mean of breast cancer was 0.484 +/- 2.5 standard deviation (Std.) compared to 0.329 +/- 0.30 Std. in normal breast tissue. In breast cancer, relative ATM expression ranged from 0.03 to 16.8 with a median of 0.57, and in normal breast it ranged from 0.093 to 1.31 with a median of 0.318. Examples of individual raw data are shown in Fig. 2. Repeat determinations from individual patients yielded reproducible results. Table II shows a subset of breast cancers and normal controls with their relative ATM expression levels, averaged from 2 independent measurements of the same sample. A mild tumor-associated increase in relative ATM transcript levels was also seen when the subgroup of matched pairs was analyzed separately. Seven of the 11 normal / cancer pairs showed a 1.2 to 2.3-fold increase in cancers compared to their adjacent normal tissue match, 3 cases were equal and only one case showed decreased (< 50%) ATM expression in the tumor. In line with the findings in primary cancers, breast cancer cell lines had even higher ATM expression with a geometric mean of 2.6 +/- 1.96 Std. and a range from 0.47 to 5.55.

We also performed a partial mutational analysis on two regions of the ATM gene (a middle region an dthe PI3 kinase region) on 8 cases of breast cancer with the highest ATM expression and found no mutations.

Key Research Accomplishments:

-Research Paper published (Kovalev S et al (2000) International Journal of Oncol 16: 825-831.

-Poster presentation at the Era of Hope Meeting, June 8-11, 2000 in Atlanta

Reportable Outcome:
1) This work was published: Kovalev S, Mateen A, Zaika AI, O'Hea BJ and UM Moll (2000) Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines. Int J Oncol 16: 825-831.

2) A repository of total RNA extracted from the 89 cases of breast cancer and 29 cases of normal breast tissue has been made an dwill be availabe for future molecular studies.

3) Based on the experience and training received from the work supported by this award, Dr. Sergey Kovalev obtained a faculty position in his native country at the University of Yekaterinburg, Russia. There, he is working in the field of molecular diagnosis of malignancies, including breast cancer.

Conclusions:
Although the ATM locus falls within a region of frequent LOH in breast and other human cancers, we did not find a reduction in ATM mRNA expression levels in our cohort of 89 sporadic breast cancers. Based on the available mutations data (mostly truncations leading to unstable protein), such a reduction would be expected at least in some cases if the ATM gene would play a causal role in breast cancer. Our study did not find evidence in support of the hypothesis that ATM is a tumor
suppressor gene causally involved in sporadic breast cancer. Our study agrees withseveral new studies in the literature which appeared since the proprosal was originally submitted. For reasons of higher relevance and pay-off, I requested a change of scope to the p73 project.

References: See reprint.
Appendices: Kovalev S, Mateen A, Zaika AI, O'Hea BJ and **UM Moll** (2000) Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines. *Int J Oncol* **16**: 825-831.

Part II: **In vivo role of the p73 gene in breast cancer**

The p73 gene is a structural and, in overexpression systems, functional p53 homolog. Ectopic p73 expression can activate a broad subset of p53-responsive genes, induce apoptosis, cell cycle arrest and growth suppression. Nevertheless, p73's role in tumorigenesis is unclear. Current genetic data exclude that p73 is a Knudson-type tumor suppressor. However, endogenous p73 is induced and activated for apoptosis by c-abl in response to cisplatin-induced DNA damage, suggesting some similarity to p53 in regulating checkpoint control pathways. Defining the upstream pathways that signal to p73 will be crucial for understanding its biological role.

In this study, we asked whether oncogenes can induce and activate endogenous p73. We show that p73 b, and to a lesser extent p73 a proteins are upregulated in p53-deficient H1299 cells in response to a panel of the overexpressed oncogenes E2F1, cMyc and E1A. The oncogene-mediated p73 accumulation is stronger than the p73 response after cisplatin, which is restricted to p73 a and occurrs only in one of two cell lines tested. E2F1, cMyc and E1A-

mediated p73 upregulation leads to the activation of p73 transcription function, as shown by the induction of the endogenous p73 target proteins p21 and HDM2, and by p73-responsive reporter activity which is inhibited by a dominant negative mutant of p73 (p73DN). Moreover, oncogene-mediated activation of endogenous p73 induces apoptosis in Saos2 cells, which is largely abrogated by p73DN, indicating p73 dependence. In contrast, in stable H1299 clones that overexpress cMyc, p73 protein accumulates but largely fails to activate a reporter and to induce endogenous response genes. The latter indicates that in p53-deficient tumor cells with activated oncogenic pathways, clonal outgrowth favors loss of p73 function. Taken together, this data shows that oncogenes can signal to p73 *in vivo*. Moreover, our data provide a mechanism for the fact that a broad spectrum of human tumors, with their frequent deregulation of oncogenes such as E2F1 and myc, overexpress p73 compared to their normal tissue of origin.

Statement of Work: Task 1A. To identify if downregulation of p73 with an antisense strategy alters growth rate of breast cancer cells months 1-9). This section using antisense downregulation was not done because of technical difficulties. We were unable to identify antisense oligonucleotides that effectively downregulated endogenous p73 mRNA.

Task 1B. Determine if cellular oncogenes induce p73 protein(s) .

Results

Endogenous 73 a and b proteins are induced in response to cellular and viral oncogenes E2F1, cMyc and E1A. The majority of functional and regulatory p73 studies to date used ectopically expressed p73 proteins. To reliably detect endogenous p73 proteins, we used 3 different p73 specific antibodies. They comprised the p73 b-specific monoclonal GC15, a p73 a-specific polyclonal raised against a C-terminal peptide (poly p73 a) and a pan p73 polyclonal raised against an N-terminal peptide (poly p73 N). Using transfected p73 a and b as positive controls (Fig 1, lanes 2 and 7), the antibodies recognize endogenous p73 a and b in several tumor cell lines including the p53-deficient human H1299 line (Fig 1). H1299 cells express a basal level of p73 a and b (see lanes 1 and 9). Simian COS cells express the highest level of both isoforms (lanes 4, 5 and 8) in contrast to SK-N-AS cells which express no detectable p73 b (lane 6) and only minute amounts of p73 a (lane 3), consistent with previous reports (19).

Next we tested whether viral and cellular oncogenes, which are major upstream signals for p53 activation, are also physiologically relevant upstream signals for endogenous p73 induction. H1299 cells were transiently transfected with various oncogene-expressing plasmids and expression was verified by immunoblotting with their respective antibodies (Fig 2A). As seen in Fig 2B and C, both p73 a and b proteins were markedly induced after expression of E2F1, cMyc or adenoviral E1A when compared to empty vector (see vimentin for equal loading). Since the transfection efficiency, as judged by cotransfected GFP, varied between 50 - 70%, a slight variation in fold induction from experiment to experiment was observed. A representative experiment is shown in Figs 2B and C, where p73 a and p73 b induction ranged from 1.9 - 3.7-fold compared to cells transfected with empty vector. P73 b reproducibly was induced to a greater degree than p73 a (2.3 - 3.7-fold versus 1.9 - 2.0-fold in this experiment).

Cisplatin (CDDP, 25 mM exposure for 24 h) was recently found to induce endogenous p73 in the human colon carcinoma cell line HCT 116-3(6) and in mouse embryo fibroblasts

(MEF) (37). The latter report did not specify which p73 isoform was induced (37). Cisplatin-induced p73 accumulation was due to posttranslational induction rather than an increase in p73 mRNA, analogous to the mechanism of p53 induction by DNA damage and oncogenic stress (37). When we treated H1299 and the human diploid fibroblast line MRC5 with cisplatin under the same conditions (CDDP, 25 mM for 24 h), p73 was responsive, albeit only to a moderate degree and in a cell type specific manner. Fig 3 shows that MRC5 cells showed a 1.4-fold induction of p73 a but not p73 b, while H1299 cells failed to respond completely. In contrast, p53 was induced 3.6-fold in MRC5 cells by this treatment. Taken together, we conclude that oncogenes induce p73 more potently than cisplatin and that they induce both isoforms.

Oncogene-mediated p73 induction leads to activation of p73 response genes. P73 shares many response genes with p53 *in vivo*. This has been shown in several cell systems using transient or inducible p73 expression (1, 8, 9). For example, p73 induces p21 and HDM2 mRNAs by Northern blot analysis, although somewhat less efficiently than p53 (9). We therefore tested whether the oncogene-mediated induction of endogenous p73 also translates into transcriptional activation by p73. When p53-deficient H1299 cells were transiently transfected with expression plasmids for E2F1, cMyc and E1A, endogenous p73 protein was upregulated (Fig 4A, top panel, compare with empty vector) and this upregulation was accompanied by the induction of the p73 response gene products p21 and HDM2 (Fig 4A, middle panels). These two response genes are direct *in vivo* targets of p73 a and b, as shown by ectopic p73 inducibly expressed in p53-deficient EJ and H1299 cells (9, 36). Oncogene expression was confirmed by immunoblots (data not shown). E2F1 reproducibly caused a stronger transactivation of the p21 and HDM2 genes than cMyc and E1A. A representative example is shown in Fig 4A with a 4.0-fold induction of p21 and a 1.7-fold induction of HDM2. We reason that in p53-deficient H1299 cells p73 substitutes for p53. In p53 expressing cells, transactivation of mdm2 in response to a broad spectrum of overexpressed oncogenes including the panel used here has been shown to be indirect and strictly dependent on p53 (for review see Ref. 47). Transactivation of p21 by cMyc and E1A is also p53-dependent, although E2F1, in addition to activating the p21 promoter through p53, can transactivate p21 directly (48). Taken together, this data strongly suggests that with the partial exception of E2F1, the induction of HDM2 and p21 after oncogenes is mediated through p73 in these p53-defocoent cells.

The oncogene-mediated activation of the p73 transcription function was further supported in luciferase reporter assays of H1299 cells using the p73 responsive PG13-Luc reporter. As shown in Fig 4B, E2F1 was the strongest activator of p73 while the other oncogenes showed lower but significant activity compared to vector controls. P73 b exhibited 80% of the activity of p53 (on a molar basis) in transactivating the PG13-Luc reporter (data not shown). To confirm that the oncogene-induced PG13 reporter activity is mediated through p73, we tested the effect of a co-expressed dominant negative mutant of p73 (Fig 4C). P73DN encodes aa 345-636 of human p73 a and acts as a dominant negative mutant by inhibiting p73 a-dependent transactivation (Fig 4C) but does not interfere with p53-dependent transactivation. This mutant also binds to p73 a and b *in vitro* and *in vivo* but not to p53 (M Irwin and WG Kaelin, to be published elsewhere). When co-expressed with E2F1, E1A and cMyc, the p73DN mutant suppressed reporter activity by 44% (E2F1), 58% (E1A) and 35% cMyc. A very similar suppressive effect was seen with p73DNs (aa 313-404) (data not shown). Taken together, we

conclude that oncogenes induce and activate endogenous p73 for transactivation of effector genes.

Oncogene-mediated activation of endogenous p73 induces apoptosis in p53-deficient tumor cells. The activation of the transcription function of p73 by oncogenes also suggested that these upstream signals might mediate the activation of the apoptotic function of p73. To test this prediction, we performed apoptosis assays on transiently transfected Saos-2 cells with or without co-expressed p73DN using the in situ TUNEL assay. As seen in Fig 5, expression of E2F1, cMyc and E1A in p53-deficient Saos-2 cells induces apoptosis that resembles the one seen with transfecting p73 b directly. Importantly, the apoptotic avtivity of each oncogene was greatly suppressed when co-expressed with p73DN (84 % for E2F1, 96% for cMyc and 72% for E1A). Taken together, this data shows that oncogene-mediated apoptosis in p53-deficient tumor cells depends on p73 function.

Stable H1299 clones overexpressing cMyc have upregulated p73 protein levels but favor selection for loss of p73 function. Our previous results on transiently transfected H1299 and Saos-2 cells indicates that, in the absence of p53, overexpressed oncogenes are able to activate the transcriptional and apoptotic activity of p73. From this data one might predict that clonal outgrowth of p53-deficient tumor cells with stable overexpression of oncogenes selects for loss of p73 transactivation function. To test this hypothesis, vector control and cMyc transfected H1299 cells (1x107 cells seeded) were selected in G418 for 3 weeks. Surviving foci were then ring cloned and expanded into sublines. Of the only 22 surviving cMyc foci, 7 were randomly picked and successfully established. As shown in Fig 6A, all clones overexpressed cMyc, albeit to various degrees compared to vector control. Cell extracts were then probed for p73 protein levels (Fig 6B, top 2 panels). As already seen with transient cMyc transfections, p73 a and b were found to be markedly induced in all subclones (Fig 6B; fold inductions compared to vector alone are indicated). However, in 6 of the 7 subclones upregulated p73 protein failed to induce p21 protein above baseline as present in the vector control (Fig 6B, third panel). Moreover, 3 of the 7 clones failed to induce significant levels of HDM2. Although clones 3 and 5 did show increased HDM2 levels, they are likely to be unrelated to p73 since these clones did not induce p21 concordantly. Only clone 7 exhibited concordant p21 and HDM2 induction. Interestingly, this was the only one among the seven clones with extremely slow growth, while all others grew at a much faster and relatively similar rate. It is therefore possible that p73 function in clone 7 is retained but exerts a strong negative effect on the cell cycle.

The lack of p73 transcriptional activity in these cMyc-expressing stable subclones was further confirmed in luciferase reporter assays. As shown in Fig 6C, transiently transfected p73 b gave a strong response. In contrast, our panel of cMyc clones exhibited no significant transactivation activity with reporter levels comparable to the empty vector clone, with the possible exception of clone 7. This clone showed a marginal p73 reporter activity consistent with its effects on endogenous target genes (see Fig 6B) and its extremely slow growth. Of note, by Western blot analysis with polyclonal anti-p73 a and monoclonal GC15 antibody we did not detect any aberrant p73-related polypeptides or a form consistent with delta Np73 in these cMyc clones. This suggests that the accumulated p73 proteins might harbor missense or small deletion mutations, and mutational analysis of these clones is underway. Taken together, our results show that clonal outgrowth tends to select for loss of p73 function. Conversely, cells with

constitutive activation of functional p73 appear to be largely unable to successfully establish stable clones. We conclude that in p53-deficient tumor cells with activated oncogenic pathways, p73 appears to exert an important suppressor function *in vivo*.

Key Research Accomplishments:
-Research Paper published (Zaika et al *J Biol. Chemistry* **276**:11310-11316, 2001.

- August 2000 Cancer Genetics &Tumor Suppressor Genes, Cold Spring Harbor Symposium, NY "Oncogenes induce and activate endogenous p73 protein"

Reportable Outcome: Zaika AI, Irwin M, Sansome S, **UM Moll** (2001) Oncogenes Induce and Activate Endogenous p73 Protein. *J Biol. Chemistry* **276**:11310-11316.

Conclusions: This data shows that oncogenes can signal to p73 *in vivo*. Moreover, our data provide a mechanism for the fact that a broad spectrum of human tumors, with their frequent deregulation of oncogenes such as E2F1 and myc, overexpress p73 compared to their normal tissue of origin.

References: See reprint
Appendix: Reprint of published paper.

Statement of Work :Task 2. Combinatorial assays to determine if dominant negative interactions among p73 isoforms and between p73 and p53 can be demonstrated months 12-24).

Introduction
The p53 family member p73 has significant homology to the p53 tumor suppressor. Human full length p73 (TA-p73) shares 63% amino acid identity with the DNA-binding region of TP53 including conservation of all DNA contact residues, as well as 38% and 29% identity with the tetramerization domain and transactivation domain, respectively. Ectopically overexpressed TA-p73a and b (two C-terminal splice variants) largely mimic p53 activities including the induction of apoptosis, cell cycle arrest and the transactivation of an overlapping set of target genes. Moreover, deregulation of oncogenes E2F1, cMyc and E1A induces apoptosis in tumour cells in a p53-independent manner by transcriptionally inducing and activating endogenous TA-p73 proteins. Furthermore, endogenous TA-p73 is activated to mediate apoptosis by a restricted spectrum of DNA damage such as cisplatin, taxol and g-irradiation via a pathway that depends on the non-receptor tyrosine kinase c-abl. Thus, TA-p73 might function synergistically with p53 in a tumour surveillance pathway. However, despite this homology, data from human tumours and p73-deficient mice argue against a classical Knudson-type tumour suppressor role for the TP73 gene. TP73-deficient mice lack a spontaneous tumour phenotype and inactivating mutations in human tumours are extremely rare (over 900 tumours analyzed to date). Moreover, while all normal human tissues studied express very low levels of p73, multiple primary tumour types and tumour cell lines overexpress p73, including cancers of the breast, lung, esophagus, stomach, colon, bladder, ovary, liver, bile ducts, ependymal lining, myelogenous leukemia and neuroblastoma. To date, most studies identifying p73 overexpression in primary human tumours have examined total levels of p73 with a few exceptions that specifically measured TA-p73 or Ex2Del p73. Importantly, in mouse, an

N-terminally truncated DNp73, generated from an alternative promoter in Intron 3, plays an essential anti-apoptotic role during p53-driven developmental neuronal death *in vivo* by acting as a dominant negative inhibitor of p53. We therefore sought the human counterpart of DNp73 and examined its potential role in cancer.

The human TP73 gene can produce DNp73

Mouse DNp73 differs from TA-p73 by a novel Exon 3', which replaces the first 3 Exons, and is spliced in frame to Exon 4 of the TP73 gene[11]. By sequence alignment of a human genomic BAC clone containing TP73 (GenBank Accession Nr. AL 136528), we identified a region with 77% identity to the 5'UTR of mouse DNp73 mRNA (Genbank Accession Nr. Y 19235). This allowed us to predict the human Exon 3' and design isoform-specific primers for human DNp73. Full length DNp73a cDNA, spanning Exons 3'-14 and including 220 bases of 5'UTR and 103 bases of 3'UTR, was cloned by RT-PCR from total RNA of human placenta and MDA 231 breast cancer cells and sequence confirmed. Human Exon 3' consists of 13 unique amino acids with almost complete identity to mouse Exon 3' (12 of 13 residues are identical). Human Intron 3 contains the predicted TATA box 30 nt upstream of the transcriptional start site, which is located 7.6 kb downstream of Exon 3.

Analysis of DNp73 and TA-p73 expression in human tumours

Unique cDNA primers were designed for the specific amplification of DNp73 from tissues by semiquantitative RT-PCR. We then determined the expression levels of DNp73 in 52 breast cancers and compared them to 8 unrelated normal breast tissues. All but one normal breast tissues showed either non-detectable or very low levels of DNp73. In contrast, 16 of 52 breast cancers (31%) expressed DNp73 levels that were between 6 and 44 - fold higher than the normal tissue average. Since we previously showed that breast cancers also overexpress TA-p73[14], we next used isoform-specific semiquantitative RT-PCR to measure DNp73 and TA-p73 simultaneously. Among these 16 cancers with a 6 to 44-fold increase of DNp73, 12 cancers showed much higher upregulation of DNp73 than of TA-p73 (data not shown).

We next analyzed a spectrum of tumours that were matched with the patients' normal tissues of origin. Of 16 matched cancer pairs (ovarian, breast, cervix, kidney and colon cancer) and 1 large benign ovarian tumour (serous cystadenoma), DNp73 was specifically upregulated 3 to 78-fold in 10 tumours (63%), while TA-p73 was upregulated 3 to 155-fold in 7 tumours (41%) (third column), compared to their respective normal tissues of origin. Importantly, when upregulation of DNp73 and TA-p73 in a given tumour is analyzed more closely, in 7 of the 10 tumours (70%) DNp73 is upregulated disproportionately to a far greater degree than TA-p73 upregulation (fourth column). These include 4 ovarian tumours, 2 breast cancers and 1 cervical cancer. Their excessive rise in DNp73 compared to their rise in TA-p73 ranges from 5 to 16-fold. Four cancers within this group exhibited exclusive upregulation of DNp73 (tumours Nr. 5, 6, 8 and 10). Only 2 tumours exhibited an inverse ratio with an excessive TA-p73 rise compared to their DNp73 rise (tumours Nr. 3 and 7). One additional tumour (Nr. 9) with concomitant upregulation of both isoforms could not be quantitated because the corresponding normal tissue levels were undetectably low (ND). Furthermore, among the remaining 7 tumours that did not upregulate DNp73, 2 of 4 tumours that we analyzed (Nr. 11 and 17) showed tumour-specific upregulation of Ex2Del p73 instead of DNp73. Both of those tumours also failed to upregulate TA-p73. Ex2Del p73 is a dominant negative isoform of p73 lacking the transactivation domain, which is

generated from the TA promoter by splicing out Exon 2. It has been previously shown to be upregulated in some ovarian cancers and breast cancer cell lines1[1,15,17]. Thus, a total of 9 tumours in our series (53%) exhibit either exclusive or excessive upregulation of dominant negative p73. Moreover, of the 14 tumours available for p53 mutational analysis by immunocytochemistry, 10 tumours had undetectable p53 levels, suggesting wild-type status, while 4 tumours showed nuclear overexpression, suggesting p53 mutation. This estimated mutational rate (29%) is in good agreement with the reported rates of p53 mutations in these tumor types (about 30 %)[18]. Thus, it appears that in a total of 8 analyzable tumours with disproportional upregulation of a dominant negative p73 isoform (7 tumours with DNp73 and 1 tumour with Ex2Del), 7 tumours likely harbored a wild type p53 genotype, while only 1 tumour exhibited a concomitant p53 mutation (fifth column).

DNp73 is an Efficient Dominant Negative Inhibitor of wild-type p53 and TA-p73 function
To test the hypothesis that human DNp73 is a dominant negative inhibitor of human wild-type p53 and TA-p73, we first performed reporter assays with expression plasmids for wild-type p53, TA-p73a and b and a p53/TA-p73-responsive Luciferase reporter in the presence or absence of DNp73a in p53 *null* H1299 cells and SaOs2 cells . DNp73a exhibited a dose-dependent, complete suppression of the transcriptional activity of wild-type p53 and TA-p73a and b. Moreover, a molar ratio of 1:1 between DNp73a and wild-type p53 potently inhibited p53 activity, yielding a reduction by 92%. In comparison, a 1:1 molar ratio reduced TA-p73b activity by 62%, although a 3-fold molar excess of DNp73a completely blocked TA-p73b activity (97% reduction). The latter suggests that DNp73a is a stronger inhibitor of wild-type p53 than of TA-p73b. Furthermore, DNp73a also efficiently suppresses endogenous target gene products of wild-type p53 and TA-p73. In HeLa and H1299 cells, transfection of wild-type p53 or TA-p73 induces endogenous HDM2, 14-3-3s and p21Waf1 compared to basal levels seen with empty vector. However, the concomitant expression of DNp73a strongly suppresses each of these response gene products.

Moreover, DNp73a is a strong inhibitor of apoptosis induced by wild-type p53 and TA-p73. HeLa and SaOs2 cells undergo wild-type p53- and TA-p73 dependent cell death as assessed by Annexin V staining and TUNEL assay. This apoptotic activity is completely abolished by co-expression of DNp73a (Fig. 4a and data not shown). The inhibitory action of
DNp73a is dependent on the presence of transcription-competent wtp53 and TA-p73, since DNp73a alone cannot affect apoptosis. Furthermore, in agreement with the above results, DNp73a is an inhibitor of colony suppression mediated by wild-type p53 and TA-p73. Reintroduction of wild type-p53 and TA-p73 suppresses growth of SaOs2 cells[2,19] and this suppression is thought to be largely due to apoptosis[20]. In keeping with these results, transfection of wild-type p53 strongly suppresses macroscopic colony formation of SaOs2 cells compared to many visible colonies with vector backbone alone (4 foci for wild-type p53 versus 1778 foci for vector control). In contrast, co-expression of DNp73a together with wild-type p53 at a 1:1 molar ratio counteracts this effect, leading to a 12.5-fold increase in the number of colonies from 4 to 51. Likewise, TA-p73a, although not quite as potent as wild-type p53, suppresses colony formation (82 foci)[2], but co-expression of DNp73a together with TA-p73a again antagonizes this effect and increases the number of macroscopic colonies by 8.1-fold to 669 foci. The higher rescue ability of DNp73a with respect to wild-type p53 is reminiscent of its stronger inhibition of wild-type p53-mediated transactivation compared to TA-p73 -mediated transactivation . Entirely consistent with this

finding were data from a subsequent p53 expression analysis of surviving colonies. A complete loss of p53 protein expression was found in 2 of 2 randomly picked and expanded colonies that were derived from plates transfected with wild-type p53 alone. This is in agreement with the fact that wild-type p53 expression is incompatible with the outgrowth of colonies in such an assay and the rare colonies that do grow escape because they have lost wild-type p53 expression[21]. In contrast, all 3 randomly picked colonies from plates cotransfected with wild-type p53 and DNp73a had detectable levels of p53 protein, indicating that DNp73a neutralizes the growth suppressive effect of wild-type p53, thereby removing the selection pressure to delete the wild-type p53 plasmid. Thus, DNp73a is able to counteract p53 and TA-p73 – induced colony suppression in transformed human cells.

DNp73 inhibits wild-type p53 and TA-p73 function by heterocomplex formation

One explanation for this dominant negative effect is a direct physical interaction between DNp73 and either wild-type p53 or TA-p73 proteins, analogous to the dominant negative mode of action of mutant p53 proteins towards wild-type p53. To test this hypothesis directly, lysates prepared from p53 *null* SaOs2 cells cotransfected with wild-type p53 and DNp73a were immunoprecipitated with monoclonal antibody, ER15, which recognizes DNp73a. Immunoblot analysis with an antibody specific for p53 (CM1) revealed a complex of the 2 proteins. As a control, no such complex was seen in SaOs2 cells transfected with DNp73a alone and immunoprecipitated with ER15, indicating the specificity of the detection. Of note, TA-p73 isoforms are unable to form a protein complex with wild type p53[16,22-24], excluding the possibility that the observed p53 band was co-immunoprecipitated via the endogenous TA-p73 protein of SaOs2 cells. Moreover, a similar complex was seen in wild-type p53 expressing human U2OS cells after transfection with DNp73a alone. No such complex is seen when an irrelevant monoclonal antibody against green fluorescent protein (GFP) is used. The same specific complex can again be immunoprecipitated from U2OS cells using a monoclonal antibody specific for p53 (421) and immunoblotted with a polyclonal antibody specific for DNp73 that does not crossreact with any TA-p73 proteins. Again, no such complex is found with preimmune mouse IgG.

Conclusion

The p53 family member p73 has significant homology to p53, but tumour-associated upregulation of p73 and genetic data from human tumours and p73-deficient mice rule out a classical Knudson-type tumour suppressor role. We report that the human TP73 gene gives rise to an N-terminally truncated isoform, DNp73, which is derived from an alternative promoter. DNp73 is frequently overexpressed in a variety of primary human cancers with upregulated p73. Functional studies indicate that DNp73a, which lacks the transactivation domain of full length p73 (TA-p73), acts as a dominant negative inihibitor of both wild-type p53 and transactivation-competent TA-p73. Moreover, DNp73a counteracts apoptosis and tumour cell growth suppression induced by wild type p53 and TA-p73. The underlying mechanism of inhibition is heterocomplex formation between DNp73a and e.g. wild type p53. Thus, DNp73 mediates a novel inactivation mechanism of wild-type p53 and TA-p73 via a dominant-negative family network. Increased expression of DNp73 appears to bestow oncogenic activity upon the TP73 gene, a trait that is selected for in human cancers.

Key Research Accomplishments:

The p53 family member p73 has significant homology to p53, but tumour-associated upregulation of p73 and genetic data from human tumours and p73-deficient mice rule out a classical Knudson-type tumour

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Reportable Outcome:

- Alex I. Zaika, Neda Slade, Christine Sansome, Troy Joseph, Mike Pearl, Eva Chalas and **Ute M. Moll.** DNp73, a Dominant Negative Inhibitor of wild-type p53 and TA-p73, is Upregulated in Human Tumours. *Journal of Experimental Medicine, in press (scheduled appearance mid Sept 2002).*

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Appendix: see preprint.

In addition, I published an invited Review Article on the p53 gene family:

Moll UM, Erster S, Zaika A. p53, p63 and p73 ? solos, alliances and feuds among family members. Biochim Biophys Acta. 2001 Dec 28;1552(2):47-59.

Appendix: see reprint

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Review

p53, p63 and p73 – solos, alliances and feuds among family members

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Abstract

p53 controls crucial stress responses that play a major role in preventing malignant transformation. Hence, inactivation of p53 is the single most common genetic defect in human cancer. With the recent discovery of two close structural homologs, p63 en p73, we are getting a broader view of a fascinating gene family that links developmental biology with tumor biology. While unique roles are apparent for each of these genes, intimate biochemical cross-talk among family members suggests a functional network that might influence many different aspects of individual gene action. The most interesting part of this family network derives from the fact that the p63 and p73 genes are based on the 'two-genes-in-one' idea, encoding both agonist and antagonist in the same open reading frame. In this review, we attempt to present an overview of the current status of this fast moving field.

Keywords: p53; p63; p73; Transcription factor; Tumor suppressor gene; Oncogene

1. Introduction

p53 controls a powerful stress response by integrating upstream signals from many types of DNA damage and inappropriate oncogenic stimulation, all of which lead to p53 activation. Activated p53 elicits apoptosis, cell cycle arrest and, in some circumstances, senescence, thereby preventing the formation of tumors. Hence, loss of p53 function is a preeminent finding in most human cancers, whether directly through mutation of p53 itself, the most common mechanism [1], impaired nuclear retention of p53 [2,3], loss of the upstream activator p14ARF through silencing or mutation [4], or amplification of the p53 antagonist HDM2 [5].

Despite the central role of p53 in tumorigenesis – and an intense search by many laboratories – no related genes were found for 20 years. This changed suddenly in 1997, when two novel family members were identified and termed p73 [6] and p63 [7–11]. (Since p63 was cloned independently by multiple groups, it led to a prolific and rather confusing nomenclature: KET, p51A and p51B, p40, p73L, NBP.) Based on their remarkable structural similarity to p53, p63 and p73 generated instant excitement and quick expectations about their biological functions. Four years later we have unearthed striking similarities but also surprising diversities. Both genes give rise to proteins that have (i) entirely novel functions, and, (ii) p53-related functions. However, the latter are complex because they can be of an agonistic or antagonistic nature. Both p63 and p73 share over 60% amino acid identity with the DNA-binding region of p53 (and even higher identity amongst themselves), including conservation of all DNA con-

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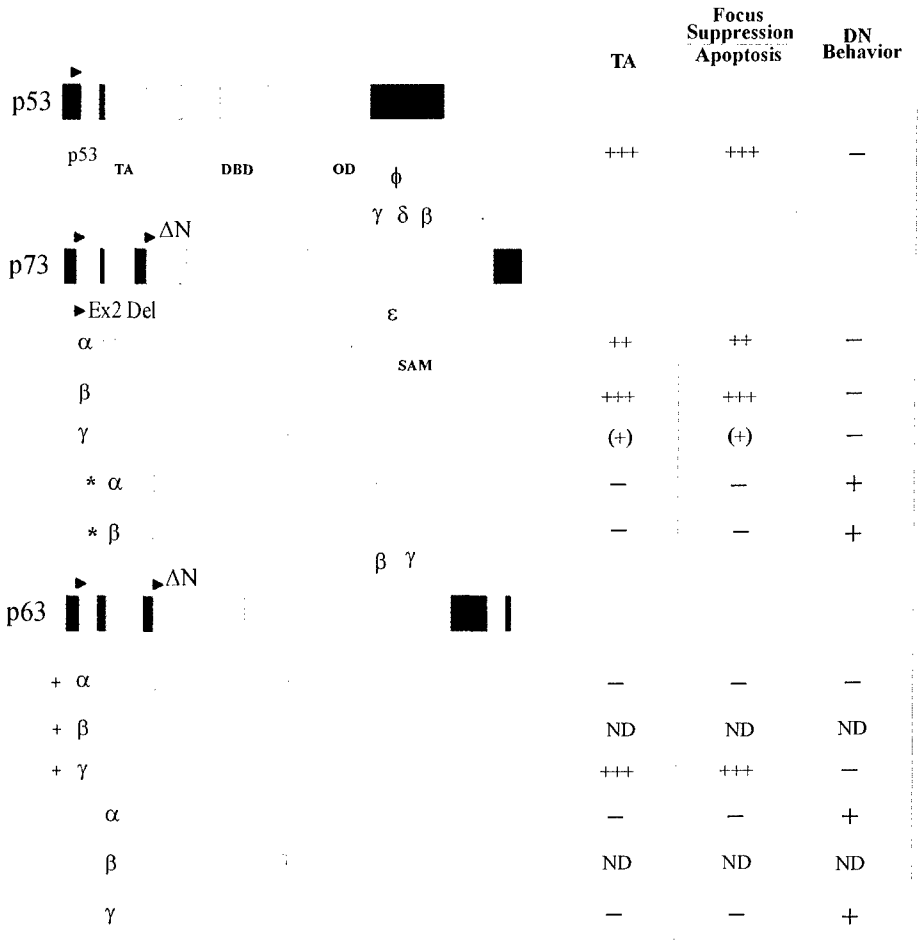

Fig. 1. Gene architecture of the p53 family. In contrast to the simple structure of the p53 protein which harbors the TA, the DBD and the OD as the three major modules, the products of TP73 and TP63 are complex and can contain a C-terminal SAM domain. Both genes contain two promoters. The P1 promoter in the 5′UTR region produces full length proteins containing the TA domain, while the P2 promoter in Intron 3 produces TA-deficient proteins with dominant negative functions towards themselves and towards p53. In addition, extensive C-terminal splicing and, in the case of TP73 another N-terminal splice variant Ex2Del, further modulate the p53-like functions of the TA-proteins (see columns).

tact and structural residues that are hotspots for p53 mutations in tumors. Even the conditional temperature-sensitive residue Ala143 in p53 is preserved in p63 and p73 [12]. In addition, p73 shows 38% identity with the tetramerization domain of p53 and 29% identity with the transactivation domain (TA) of p53. In vertebrates, the p73 and p63 genes are ancestral to p53 and possibly evolved from a common p63/p73 archetype [6,7].

2. Gene architecture of the p53 family

In sharp contrast to the simple gene structure of p53, which is highly conserved from mollusk to human, the structures of the p63 and p73 genes are complex (Fig. 1). Human TP53 has a single promoter which encodes a single protein of 393 amino acids. The three most conserved domains of p53 are the transactivation domain (TA), the specific DNA-binding domain (DBD) and the oligomerization domain (OD). In contrast, TP63 and TP73 make heavy use of an alternative promoter and alternative splicing (Fig. 1). TP63 and TP73 each have two promoters, P1 in the 5′UTR upstream of a non-coding exon 1, and P2 located within the 23 kb spanning Intron 3. P1 and P2 promoters produce two diametrically opposed classes of proteins: those containing the TA

(TAp63, TAp73) and those lacking it (ΔNp63 and ΔNp73).

TA-proteins can mimic p53 function in vitro and in cell culture including transactivating many p53 target genes and inducing apoptosis, while ΔN-proteins act as dominant negative inhibitors towards themselves and towards other family members [4,7,13]. Moreover, TP73 can undergo alternate splicing of Exon 2 which again produces a TA-deficient protein called p73 Ex2Del. As if this complexity were not enough, TP73 and TP63 also undergo multiple C-terminal splicing, skipping one or several exons (six forms for TP73 (called α, β, γ, δ, ε and φ with α being full length) [6,14,15] and three forms for TP63 (α, β and γ) [7]. In some isoforms, exon splicing also leads to unique sequences due to ensuing frameshifts. Splicing of different 'tails' further modulates the p53-like function of TA-proteins. Importantly, in all C-terminal splice and ΔN forms, the DBD and tetramerization domain are still preserved. Structurally, the γ forms of TP73 and TP63 most closely resemble p53 itself, featuring just a small C-terminal extension beyond the last 30 amino acid stretch of p53. Surprisingly, though, while TAp63γ (also called p51A) is as powerful as p53 in transactivation and apoptosis assays [7], TAp73γ is rather weak. The α forms of TP73 and TP63 contain an additional highly conserved SAM motif (sterile α motif). SAM motifs are protein interaction modules found in a wide variety of proteins implicated in development. The crystal and solution structure of p73 SAM agree with each other and feature a five-helix bundle fold that is characteristic of all SAM domain structures [16,17]. Other SAM-containing proteins are e.g. the ETS transcription factor TEL that plays a role in leukemia, the polycomb group of homeotic transcription factors and the ephrin receptors. Despite predictions of homo- and hetero-oligomerization of SAM-containing proteins, though, p73 SAM appears monomeric by experimental analysis, casting doubt whether this domain mediates interaction of p73 with heterologous proteins [17]. There are also functional differences between TAp73α and TAp63α. While TAp73α is comparable to p53 in transactivation and apoptosis assays, TAp63α (also called p51B) is very weak [7]. The reason for this difference is not obvious from the structure and remains unclear. In general, more structure–function analysis is needed to understand why and how C-terminal variations influence function.

The P2 promoters transcribe truncated forms of TP73 and TP63 that lack the TA. While ΔNp63 has been shown to occur in human and mouse, ΔNp73 has so far only been reported in mouse. Recently we have also cloned ΔNp73 from human tissues (A. Zaika and U.M. Moll, unpublished results). Most importantly, these ΔNp73 and ΔNp63 proteins behave in a dominant negative fashion towards their own TA-proteins and towards p53 in vivo in the mouse and in transfected human cells. Strikingly, squamous cell carcinoma of the skin produces high levels of ΔNp63α. Moreover, the TP63 locus was contained within a frequently amplified region in this cancer type [18]. Furthermore, ΔNp73 is the predominant TP73 product in the developing mouse nervous system [4,13] (see below).

In summary, by using alternate promoters and exon splicing, TP73 and TP63 genes can generate an impressive modular complexity by combining a specific 'head' with a particular 'tail'. In practice, this means that our understanding of their biological roles will greatly depend on knowing which forms get expressed under what circumstances.

3. TP63 and TP73 play important roles in development and differentiation

Both genes play important and, despite their structural similarity, surprisingly unique roles in mouse and human development. This is powerfully revealed by the striking developmental phenotypes of p63- and p73-deficient mice [4,19,20].

3.1. TP63

TP63 expression is absolutely essential for limb formation and epidermal morphogenesis (integument, tongue) including the formation of adnexa (teeth, hair, mammary and prostate glands, sweat and lacrimal glands). p63 null animals show severe limb truncations or even absence of limbs as well as craniofacial malformations. The animals do not survive beyond a few days postnatally. Reminiscent of the knock-out phenotype in mice, heterozygous germ line mutations of p63 in humans cause the rare au-

tosomal dominant developmental disorder EEC (Ectrodactyly, Ectodermal Dysplasia, Facial Clefts). The p63 mutations found in EEC patients are typically missense mutations within the DBD. These EEC mutations inhibit DNA binding of the TAp63 forms. Conversely, EEC mutations in ΔNp63 proteins cause a loss of their dominant negative properties towards p53 and TAp63γ [21]. Importantly, basal cells of normal human epithelium including the epidermis strongly express p63 proteins, predominantly the ΔNp63 isotype [7], but lose them as soon as these cells withdraw from the stem cell compartment [22]. Consistent with this notion, keratinocyte differentiation is associated with the disappearance of ΔNp63 isotypes [23,24]. Together, these data clearly establish a fundamental role of p63 in the biology of the keratinocyte stem cell and the apical ectodermal ridge of the limb bud [22]. This role is likely one in stem cell self renewal rather than in stem cell differentiation into stratified epithelium, although this remains a matter of controversy [19,20]. What appears clearer is that p63 is probably not simply required for the proliferative capacity of stem cells, since their immediate progeny, the TAC cells, are equally proliferative but have already lost p63 expression [22].

3.2. TP73

TP73 has varied but distinct developmental roles. TP73 expression is required for neurogenesis of specific neural structures, for pheromonal signaling and for normal fluid dynamics of cerebrospinal fluid and the respiratory mucosa [4]. p73 null animals exhibit highly site-specific hippocampal dysgenesis. The hippocampus is central to learning and memory and continues to develop throughout adulthood. The basis of hippocampal dysgenesis in p73-deficient mice is the selective loss of large bi-polar neurons called Cajal-Retzius located in the marginal zone of the cortex and the molecular layers of the hippocampus. These CR neurons co-express p73 and the secretory glycoprotein reelin, which is essential for neuronal migration in the cortex [4]. In addition, p73 null mice suffer from hydrocephalus, probably due to hypersecretion of cerebrospinal fluid by the choroid plexus, and from purulent pan-infections of the respiratory mucosa, likely due to mucus hypersecretion. Moreover, the animals show abnormal reproductive and social behavior which in rodents is governed by pheromone sensory pathways. This abnormality could be related to dysfunction of the vomeronasal organ which normally expresses high levels of p73.

3.3. Role of ΔNp73 in mouse development

ΔNp73 is the predominant form in the developing mouse brain [7,13]. In situ hybridization reveals strong ΔNp73 expression in E12.5 d fetal mouse brain in the preplate layer, bed nucleus of stria terminalis, choroid plexus and the preoptic area [4]. Moreover, it is the only form of p73 found in mouse brain and the sympathetic superior cervical ganglia in P10 neonatal mice [13].

Functional studies and knock-out mice showed that ΔNp73 plays an essential anti-apoptotic role in vivo. ΔNp73 is required to counteract p53-mediated neuronal death during the normal 'sculpting' of the developing mouse neuronal system [13]. Withdrawal of NGF, an obligate survival factor for mouse sympathetic neurons, leads to p53 induction and p53-dependent cell death. Conversely, NGF withdrawal leads to a decrease of ΔNp73. Importantly, sympathetic neurons are rescued from cell death after NGF withdrawal when ΔNp73 levels are maintained by viral delivery. Likewise, sympathetic neurons are rescued from Adp53-mediated neuronal death by co-infected AdΔNp73. In pull-down assays, mixed protein complexes of p53/ΔNp73 were demonstrated, suggesting a biochemical basis for the transdominance. Together, these data firmly put ΔNp73 downstream of NGF in the NGF survival pathway. It also explains why p73−/− mice, missing all forms of p73 including protective ΔNp73, undergo accelerated

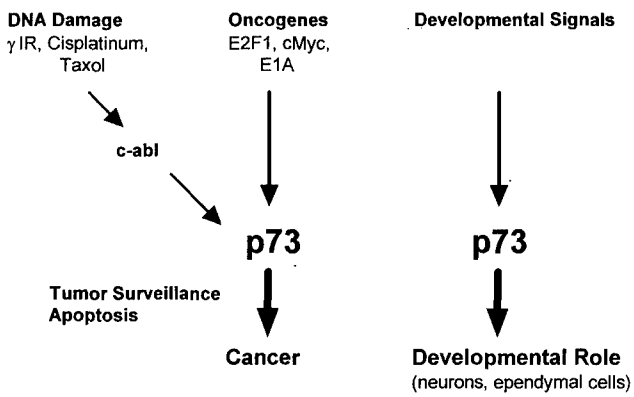

Fig. 2. Proposed model of the biological roles of p73.

neuronal death in postnatal superior cervical ganglia [13].

In tissue culture models, p73 also plays a role in differentiation of several cell lineages. TP73 expression increases during retinoic acid-induced and spontaneous differentiation of neuroblastoma cells [25,26]. Also, ectopic TAp73β, but not p53, induce morphologic and biochemical markers of neuroblastoma differentiation [25]. Moreover, expression of specific C-terminal isoforms correlate with normal myeloid differentiation. p73α and β are associated with normal myeloid differentiation, while p73 γ, δ, ε and θ are associated with leukemic blasts. In fact, p73ε is specific for leukemic blast cells [27]. Similarly, TAp73 γ and δ may play a role in the terminal differentiation of human skin keratinocytes [28]. This suggests a p73-specific differentiation role that is not shared by p53 and for the most part not shared by p63 either (see also Fig. 2).

p53 has an important developmental role in early mouse embryogenesis (E7–8 d), as revealed when the autoregulatory feedback loop with MDM2 is removed and p53 levels remain uncontrolled [29,30]. Nevertheless, in stark contrast to TP63 and TP73 null mice, TP53 null mice make it through development with essentially no problems (with the exception of rare exencephaly in females). A commonly offered explanation is that p53 functions are covered by redundant p63 and p73 functions. At least in theory, this idea could now be tested, although generating double or even triple knock-outs might be a daunting task. The concept of substitution, however, is inconsistent with the finding that ΔN isoforms, rather than TA isoforms, are the predominant proteins of TP63 and TP73 during development. Indeed, the very fact that TP63- and TP73-deficient mice have a phenotype could be viewed as evidence for the important in vivo role of ΔN isoforms during development, since conversely p53 cannot substitute for those forms.

Of note, p73-deficient mice lack spontaneous tumor formation, even after a 2 year observation period [4]. While the tumor rate after mutagenic challenge or the tumor rate of double p53/p73 null mice is currently unknown, this result is another clear difference between p53 and the other family members. It indicates that if TP73 and TP63 do have a role in tumor formation, it might be a complex one which is probably not revealed by simply eliminating the entire gene.

4. p63 and p73 expression in normal human tissues

p73 gene expression occurs at very low levels in all normal human tissues studied [26,31]. It is not readily detectable by Northern blots and immunoblots. p63, mainly its ΔN form, is readily detectable at the protein level. p63 expression is restricted to the nuclei of basal cells of normal epithelia (skin, prostate, urothelium, ectocervix, vagina) [7].

5. TAp73 and TAp63 function during forced overexpression

In general, many functional parallels are found among p53, TAp73 and TAp63 on the one hand, and between ΔNp73 and ΔNp63 on the other hand. The two groups, however, behave antithetically. Overall, far fewer studies have been reported on p63 than on p73.

When ectopically overexpressed in cell culture, p73α and β closely mimic the transcriptional activity and biological function of p53. p73β, and to a lesser extent p73α, bind to canonical p53 DNA-binding sites and transactivate many p53-responsive promoters [32–35], although relative efficiencies on a given p53 target promoter may differ from p53 and also differs among various C-terminal isoforms of TAp73 and TAp63 [34,35]. In reporter assays, p73-responsive promoters include well known p53 target genes involved in anti-proliferative and proapoptotic cellular stress responses such as p21WAF1,14-3-3σ, GADD45, BTG2, PIGs [34], ribonucleotide reductase p53R2 [36] and IGF-BP3 [37]. Bax transactivation is controversial [34,37]. TAp73α and β also induces MDM2. While there are probably still dozens of common targets that have not yet been described or discovered, it will be important to identify p73-preferred or even p73-specific targets. One example may be the Aquaporin 3 gene, a glycerol and solute transporter, which is greatly preferred by the p73β isoform compared to poor activation by p73α and p53 [38]. The physiologic significance of this relationship, however, is currently unclear. Conversely, ec-

topic p73 overexpression leads to transcriptional repression of VEGF at the mRNA and protein level, analogous to the ability of p53 to transcriptionally suppress VEGF [39].

Ectopic p73 promotes apoptosis in human tumor cell lines independent of their p53 status [6,32]. In fact, in a subset of cancer cell lines p73β is more efficient in inducing apoptosis than p53 itself [40]. Major potency differences exist among the C-terminal isoforms. Overexpression of p73 α, β and δ suppresses focus formation of p53-deficient Saos-2 cells, while p73 γ fails or suppresses only very poorly [14,32]. Similarly, TAp63α lacks significant transcriptional and apoptotic ability, while TAp63γ is very potent in both [7].

Like p53, p73 utilizes p300/CBP as its coactivator by forming a complex with the CH1 domain (aa 350–450) of p300/CBP [41]. In contrast to p53, however, p73 does not require acetylation by p300 to become transcriptionally stimulated [42].

6. Regulation of p73 and p63 protein stability and transcriptional activity

Proteasomes are implicated in the turnover of p73 proteins since proteasome inhibitors stabilize p73 isoforms. In sharp contrast to p53, however, this turnover is not mediated by MDM2. One important open question is whether an as yet undefined MDM2-like protein exists for p73, or alternatively, whether p73 stability is not specifically regulated by a dedicated E3 ligase. The molecular basis for the MDM2 resistance of p73 was found by systematic motif swapping. Region 92–112 of p53, which is absent in p73, was identified to confer MDM2 degradability to p53 [43]. p73 protein is also resistant to HPV E6, which together with E6-AP mediates hyperactive degradation of p53 in HPV-infected cells [44,45]. This relationship might have some bearing in tumors with increased p73 expression (see below). And just as MDM2 does not mediate p73 degradation, p19ARF, which stabilizes p53 levels by antagonizing the degrading action of MDM2, has not been shown to stabilize p73 protein. One potential consequence of the differential MDM2 sensitivity between p53 and p73 was seen in tissue culture: ectopic co-expression of p73 leads to a selective decrease of ectopic p53 and of endogenous induced p53, because p53 is susceptible to MDM2, while p73 is not [46]. This suggests a potential downmodulation of p53 by high levels of TAp73, an interesting family twist to keep in mind with respect to tumor formation. On a transcriptional level, however, the negative feedback regulation between the two genes is preserved. MDM2 is transcriptionally activated by p73 which, in turn, negatively regulates the transcriptional ability of p73, just as it functions towards p53 [44,47,48]. However, the mechanism is again distinct from p53. MDM2 disrupts the interaction of p73 – but not of p53 – with p300/CBP by competing with p73 for binding to the N-terminus of p300/CBP [48]. It is currently not known whether p63 has a similar relationship with p300/CBP.

Proteasome inhibitors also stabilize overall levels of p63. In an additional family twist, however, the stability of ΔNp63 isoforms may also be regulated independently of proteasomes and in fact, be promoted by physical complex formation with wild type p53. A protein–protein complex between ΔNp63α and p53, mediated by both DBDs, can form in cells. Moreover, p53 overexpression reveals p53-dependent degradation of ΔNp63 via a caspase-1-specific pathway [49]. This result may explain the observation that UV irradiation of cultured keratinocytes suppresses ΔNp63 levels [50]. A check-and-balance system may exist: while ΔNp63 is a transcriptional inhibitor of p53, p53 is a stability inhibitor of ΔNp63. This relationship also points towards another level of intimate functional cross-talk among p53 family members, a theme that will surface again and again.

7. Posttranslational modifications during activation

p53 stabilization and activation by genotoxic stress is associated with multiple posttranslational modifications at the N-and C-termini of p53 in vivo. In close temporal relationship to stress, the N-terminus undergoes heavy phosphorylation (Ser15, 20, 33, 37, 46 and Thr18, 81), which is thought to stabilize the protein by interfering with MDM2 binding, thereby disrupting the constitutively targeted degradation. The C-terminus also undergoes site-specific phosphorylation (Ser315, 392), acetylation (Lys320, 373

and 382) and sumoylation (Lys386). The C-terminal modifications are thought to activate the transcriptional activity of p53 [51]. So-called stress kinases (e.g. ATM, ATR, Chk2) which detect genotoxic stress and initiate signal transduction are in vivo kinases for specific p53 serine residues, while the histone acetyltransferases p300/CBP and PCAF (which at the same time are transcriptional coactivators) acetylate p53.

Modification differences among p53 family members are starting to be worked out. Interestingly, serine phosphorylation has not been reported for p73 and p63. Instead, p73α undergoes phosphorylation at Tyr99 by c-abl in response to γ-IR which in turn activates p73 for apoptosis [52,53]. This is due to a direct interaction between the PXXP motifs of p73 and the SH3 domain of c-abl. Interestingly, Tyr99 phosphorylation activates p73 but does not stabilize the protein. On the other hand, cisplatin also activates p73 function and stabilizes the protein but does not tyrosine-phosphorylate it. Sumoylation of C-terminal Lys627 occurs specifically in p73α but not in p73β in vitro. However, in contrast to sumoylation of p53, which activates its transcriptional activity, sumoylation of p73 promotes its degradation [54]. p63 does not have PXXP motifs and modification studies for p63 have not been reported.

8. Role of p73 and p63 in tumors

8.1. p73 is not a classic Knudson-type tumor suppressor

p73 maps to chrom 1p36.33 which frequently undergoes loss of heterozygosity (LOH) in breast cancer, neuroblastoma and several other human cancers [6]. This fact, in conjunction with the functional similarity to p53, originally led to the proposal that p73 is a tumor suppressor gene [6]. Genetic data on most cancer types – with the notable exception of leukemias and lymphomas – however, exclude p73 as a classic Knudson-type tumor suppressor, which by definition is targeted to undergo loss of expression or function during tumorigenesis. To date, in a total of over 900 primary tumors, loss of function mutations in the p73 ORF are vanishingly rare. Moreover, imprinting of the p73 locus, initially thought to be an epigenetic explanation to satisfy the 2 hit hypothesis (since it would only require one hit of LOH against the transcribed allele), is rather uncommon and if present, varies from tissue to tissue and person to person and does not correlate with p73 expression levels [15,26,55,56]. In fact, in lung, esophageal and renal carcinoma, the second p73 allele is specifically activated in tumors (loss of imprinting) [57–59].

As an additional difference from p53, p73 protein fails to be inactivated by most of the major viral oncoproteins that inactivate p53, namely SV40 T antigen [60] and Ad E1B 55 kDa [61]. For HPV E6, while clearly not inducing p73 degradation [44,45,62], controversy exists whether E6 of low and high risk strains inactivates the transcription function of p73 [62,63]. However, some viral protein products do target p73. p73 transcriptional activity is inhibited by Ad E4orf6 [64] and by HTLV 1 Tax [65]. p63 also fails to interact with SV40 T antigen and the HPV E6 protein [66].

9. Alteration of p73 expression in human cancer

Surprisingly, work from our lab and confirmed by others on multiple primary tumor types and tumor cell lines show that the most common identifiable cancer-specific alteration is an overexpression of the wild type p73 gene rather than a loss of expression [15,26]. This suggests that TP73 plays some role in tumorigenesis. To date, significant prevalence of p73 overexpression has been found in a dozen different tumor types including tumors of breast [15], neuroblastoma [26], lung [58,67], esophagus [59], stomach [68], colon [69], bladder [70,71], ovarian cancer [70% of cases in one cohort] [72–74], ependymoma [75], liver cancer [76,77], cholangiocellular carcinoma [78], CML blast crisis and acute myelogenous leukemia [27,79]. Most studies measure overexpression of full length p73 mRNA [TAp73] by RT-PCR, but a few studies also measure overexpression of TAp73 protein(s), either by immunoblot or immunocytochemistry. For example, we found overexpression of TAp73 transcripts (5–25-fold) in 38% of 77 invasive breast cancers relative to normal breast tissue, and in five of seven breast cancer cell lines (13–73-fold) [15]. Likewise, we found overexpression of

TAp73 transcripts in a subset of neuroblastoma (8–80-fold) and in 12 of 14 neuroblastoma cell lines (8–90-fold) [26]. A close correlation between p73 mRNA levels and protein levels was shown e.g. in ovarian carcinoma cell lines [72]. In a series of 193 patients with hepatocellular carcinoma, 32% of tumors showed detectable (high) p73 by immunocytochemistry and in situ hybridization, while all normal tissue had undetectable levels (low) [77]. Of note, primary tumors and tumor cell lines with p73 overexpression tend to simultaneously overexpress a complex profile of shorter C-terminal splice variants (p73γ, δ, ε, and ϕ), while the normal tissue of origin is limited to the expression of p73α and β [15]. Importantly, patients with high global p73 protein expression had a worse survival than patients with undetectable levels [77].

The single exception to this picture seems to be lymphoid malignancies. TP73 has been found to be transcriptionally silenced in some lymphoblastic leukemias and lymphomas due to hypermethylation [80,81].

Although ΔNp73 has so far only been shown in developing mouse brain [13] and human placenta (A. Zaika and U.M. Moll, unpublished results), it will be of great interest to determine whether tumors also upregulate dominant negative ΔNp73 proteins. If indeed this is the case, one could envision an important inhibitory cross-talk between ΔNp73 and wild type p53 and/or TAp73 products, thus potentially converting an anti-oncogenic synergism into an oncogenic antagonism. p73 Ex2Del might also be expressed in some tumors. Using an upstream RT-PCR primer to the 5′ UTR region, p73 Ex2Del was found to be co-expressed with TAp73 in 5 of 10 invasive ovarian cancers and in three of seven ovarian cancer cell lines. None of six normal ovarian epithelial cells or nine borderline ovarian tumors expressed p73 Ex2Del [72]. As predicted, p73 Ex2Del acts as a dominant negative inhibitor towards the transactivation and apoptotic ability of p53 and TAp73α [82].

10. Alteration of p63 expression in human cancer

Currently only a limited analysis of the expression and mutational status of p63 in primary tumors exists. The trend here appears to be rare inactivating mutations but upregulation of dominant negative forms. For example, no p63 mutations were found in 47 bladder cancers [83]. Only one missense mutation [Ala148Pro] out of 66 various human tumors and two missense mutations in 35 tumor cell lines were found [9].

The human TP63 gene is located on chrom 3p within a region which is frequently amplified in squamous cell carcinomas. Some lung cancers and squamous cell carcinomas of the head and neck show p63 overexpression associated with a modest increase in TP63 copy numbers [18]. (The authors therefore named the amplified locus Amplified In Squamous carcinoma, AIS). Importantly, although many AIS isoforms are produced in those tumors, the majority are dominant negative ΔNp63 forms (mainly p40AIS). p40AIS acts like an oncogene in nude mice and in Rat1a focus formation assays [18]. Similar findings exist in nasopharyngeal carcinoma (NPC), which almost always have functional wt p53. In 25 primary NPCs, all tumor cells overexpressed predominantly ΔNp63, which in normal nasopharyngeal epithelium is limited to proliferating basal and suprabasal cells [84]. Likewise, upregulation of ΔNp63 was found in 30 out of 47 bladder cancers [83]. Interestingly, TAp63 was concomitantly downregulated in 25 of those 47 tumors.

11. Upstream components that signal to p73 and p63

Recent data established that TP73 is able to integrate and mediate death stimuli from three different tumor surveillance pathways in vivo: oncogenes, DNA damage and T-cell receptor hyperactivation. Again, only scant data exist for TP63.

11.1. p73 is activated to mediate apoptosis by cellular and viral oncogenes

We and others recently established that the cellular and viral oncogenes E2F1, cMyc and E1A can induce and activate the endogenous TAp73α and β proteins for target gene transactivation, apoptosis and growth suppression in p53-deficient human tumor cells [85–88]. E2F1 is a direct transcriptional activator by binding to several E2F1-responsive ele-

ments within the P1 promoter of TP73 [86,88]. This is specific for TP73 since E2F1 does not activate the TP63 promoter, suggesting that this promoter is devoid of an E2F1 response element [88]. Since oncogene deregulation of E2F1 and cMyc are one of the most common genetic alterations in human tumors, these findings might provide a physiologic mechanism for TAp73 overexpression in tumors. Taken together, these data establishes another important link between p73 and human cancer.

Moreover, during E2F1-mediated apoptosis in primary mouse embryo fibroblasts (MEF) a striking non-additive cooperation between wild type p53 and p73 exists [88]. While wt MEFs show 77% apoptosis after forced E2F1 expression, p53−/− MEFs (containing p73) and p73−/− MEFs (containing p53), both show reduced killing ability after forced E2F1 expression with 12% and 15%, respectively. This excessively weakened killing ability of p73−/− MEFs, despite the presence of wt p53, is consistent with an important synergistic but independent signal emanating from TAp73 that cooperates with p53 to induce oncogene-triggered death in a tumor surveillance pathway.

11.2. An E2F1–p73 pathway mediates cell death of circulating peripheral T cells induced by T cell receptor activation

Normal peripheral T cells undergo apoptosis after hyperstimulation of their T cell receptors. This cell death pathway is mediated via the E2F1–p73 pathway [87]. Consistent with this notion, E2F1 null mice exhibit a marked disruption of lymphatic homeostasis with increased numbers of T cells and splenomegaly, suggesting that p73 plays a role in tumor surveillance pathways of lymphoid cells [89,90]. Moreover, the p73 gene is transcriptionally silenced in acute lymphoblastic leukemia and Burkitt's lymphoma due to hypermethylation [80,81,91,92]. This appears to be restricted to lymphoid tumors, since neither other hematopoietic malignancies nor solid tumors show p73 hypermethylation [80,91]. Interestingly, in radiation-induced T cell lymphomas of the mouse, the p73 locus undergoes LOH in 33% of the cases [93]. Thus, in lymphoid tumors p73 shows some genetic features of a classic tumor suppressor gene.

11.3. p73 is activated to mediate apoptosis by a restricted spectrum of DNA damage

p73 is not activated by UV, actinomycin D, doxorubicin and mitomycin C, all of which stabilize and activate p53 [6,94]. However, endogenous p73 is activated for apoptosis in response to cisplatin, taxol and γ-IR in a pathway that depends on the non-receptor tyrosine kinase c-abl [52,53,95]. Conversely, cells deficient in c-abl do not upregulate or activate their p73 and are resistant to killing by cisplatin. Together, this supports a model in which some DNA damage signals are channeled through c-abl to p73. Hence, one would predict that p73-deficient cells should have defective DNA damage checkpoint controls. This seems to be borne out by the observation that p53/p73 double null MEFs are more resistant to killing by cisplatin and Taxol than p53 single null MEFs (T. Jacks, personal communication). Endogenous p73β protein is also rapidly induced by camptothecin treatment (K. Cheng and U.M. Moll, unpublished observation). Thus, DNA-damage-dependent activation of p73 might be partly responsible for p53-independent apoptosis.

In one report, ectopic TAp63γ in a mouse erythroleukemia line is rapidly stabilized and induces WAF1 after treatment with UV, γIR or actinomycin D [96]. Surprisingly though, stabilized TAp63γ was associated with erythroid differentiation rather than apoptosis, as was seen with ectopic p53. Since ectopic TAp63γ, without additional DNA damage, caused apoptosis in baby hamster kidney cells [7], it hints at a functional versatility of TAp63γ to induce differentiation under genotoxic circumstances.

12. More transdominant cross-talk – this time between mutant p53 and TAp73 or TAp63

Physical interactions between certain – but not all – human p53 mutants and TAp73 or TAp63 proteins were found in co-immunoprecipitation assays of exogenous and endogenous proteins. Importantly, these interactions correlate with functional transdominance. In contrast, complexes between wild type p53 and p73 are not observed in mammalian cells [13,33,97]. Unexpectedly, protein contact occurs between the DBD of mutant p53 and the DBD plus

OD of p73 [98–100] rather than between the respective ODs. In co-transfections, mixed heterocomplexes were shown between p53 mutants p53Ala143, p53Leu173, p53His175, p53Cys220, p53Trp248 or p53Gly281 and TAp73 α, β, γ and δ [33,97,98,100] or TAp63 [100]. Physiologic complexes were found in five tumor cell lines between endogenous mutant p53 and p73 [97,98]. Functionally, formation of such stable complexes leads to a loss of p73- and p63-mediated transactivation and proapoptotic abilities. Moreover, E2F1-induced p73 transactivation, apoptosis and colony suppression was inhibited by co-expressed p53His175 [86]. One study proposes that the Arg/Pro polymorphism at codon 72 of mutant p53 might be a further biological determinant for binding and inactivation of p73, with 72R mutants of p53 being more inhibitory than 72P mutants [97].

This inhibition mirrors the ability of many transdominant missense p53 mutants to abrogate wild type p53 function [101,102]. It suggests that in tumors which express both TAp73 and mutant p53 (typically at very high levels due to deficient mdm-2-mediated degradation), the function of TAp73 and TAp63 might be inactivated. Moreover, these functional interactions define a network that could result in a 'two-birds-with-one-stone' effect for at least some inactivating p53 mutations. If this occurs in primary human tumors, it might have far-reaching consequences since (i) it argues for a transdominant inhibition of the tumor suppressor function of TAp73 isoforms during tumor development, (ii) it could be the underlying mechanism for the gain-of-function activity of certain p53 mutants, and (iii) it might further increase chemoresistance in cancer therapy of established tumors. p53 is exceptional among tumor suppressors in that it selects for the overexpression of missense mutants rather than for loss of expression as most other suppressor genes do. This gain-of-function results in increased tumorigenicity compared to p53 null parental cells, increased resistance to cancer agents, and increased genomic instability due to abrogation of the mitotic spindle checkpoint [103–105]. Conceivably, p63 also participates in this network. On the other hand, it should be noted that some p53 mutants clearly are recessive towards TAp73 (e.g. p53His283 and p53Tyr277) [100] and do not interfere with its action.

At least in theory, another transdominant mechanism besides hetero-oligomer formation might be promoter competition. It is conceivable that ΔNp73 or ΔNp63 homo-oligomers might have a stronger affinity to certain target gene promoters than wild type p53. In those cases, p53 inhibition would occur due to competition at the level of target gene access. Some experimental evidence does exist for this idea. In the wtp53-containing ovarian carcinoma cell line A2780, co-expression of increasing amounts of either TAp73α, β, γ or ε inhibits specific DNA binding and transcriptional activity of p53 in the absence of hetero-oligomer formation [106,107].

In short, the biological consequences of TP73 and TP63 expression might be diametrically different depending on the concomitant presence or absence of mutant p53 and/or the presence or absence of dominant negative ΔNp73 and ΔNp63 isoforms.

13. p73 and p63 appear to play a role in cancer – but as an oncogene or as a suppressor gene?

Overall, evidence is mounting that p73 does play an important role in human tumors in vivo. However, the current picture of p73's involvement in human cancer is a puzzling paradox, with the possible exception of lymphoid malignancies. How can we reconcile the p53-compensatory action of TAp73 after DNA damage or oncogene activation, which is revealed both in primary cells and p53-deficient tumor cells, with the paucity of TP73 mutations and the presence of TAp73 overexpression in many human tumors? One possible interpretation is that although p73 does mount a surveillance response, it is functionally of no consequence and therefore not targeted during tumor development. However, this view might be too simple and disregard the real possibility of 'epigenetic' TAp73 inactivation by dominant negative interference from mutant p53 and ΔN isoforms. An urgent question that needs to be answered is whether tumors upregulate dominant negative ΔNp73. If so, ΔNp73 might specifically antagonize and compromise the function of p53 and TAp73. A similar situation might exist with TP63, given the early data on ΔNp63 overexpression by head and neck squamous carcinomas [18] and nasopharyngeal carcinomas [84]. Only careful functional and biochemical analysis of such relationships will

answer the question whether p73 and p63 do matter in cancer.

14. Conclusions

Inactivation of the p53 tumor suppressor is the single most common genetic defect in human cancer. The discovery of two close structural homologs, p63 and p73, generated instant excitement and quick expectations about their biological functions. We now know that in development both genes clearly have novel, p53-independent functions. p63 is involved in epithelial stem cell regeneration and p73 in hippocampal neurogenesis, pheromonal pathways and ependymal cell function. A major future challenge is to determine what role these p53 homologs play in tumor biology. It is already clear that they are not classic Knudson-type tumor suppressors. However, the existence of inhibitory versions of both genes and intimate functional cross-talk among all family members might explain the currently conflicting properties, which argue for tumor suppressive and oncogenic roles.

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References

[1] B. Vogelstein, D. Lane, A.J. Levine, Nature 408 (2000) 307–310.
[2] U.M. Moll, M. LaQuaglia, J. Benard, G. Riou, Proc. Natl. Acad. Sci. USA 92 (1995) 4407–4411.
[3] J.M. Stommel, N.D. Marchenko, G.S. Jimenez, U.M. Moll, T.J. Hope, G.M. Wahl, EMBO J. 18 (1999) 1660–1672.
[4] A. Yang, N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, D. Caput, Nature 404 (2000) 99–103.
[5] J.D. Oliner, K.W. Kinzler, P.S. Meltzer, D.L. George, B. Vogelstein, Nature 358 (1992) 80–83.
[6] M. Kaghad, H. Bonnet, A. Yang, L. Creancier, JC. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lelias, X. Dumont, P. Ferrara, F. McKeon, D. Caput, Cell 90 (1997) 809–819.
[7] A. Yang, M. Kaghad, Y. Wang, e. Gillett, M.D. Fleming, V. Dotsch, N.C. Andrews, D. Caput, F. McKeon, Mol. Cell 2 (1998) 305–316.
[8] H. Schmale, C. Bamberger, Oncogene 15 (1997) 1363–1367.
[9] M. Osada, M. Ohba, C. Kawahara, C. Ishioka, R. Kanamaru, I. Katoh, Y. Ikawa, Y. Nimura, A. Nakagawara, M. Obinata, S. Ikawa, Nat. Med. 4 (1998) 839–843.
[10] B. Trink, K. Okami, L. Wu, V. Sriuranpong, J. Jen, D. Sidransky, Nat. Med. 4 (1998) 747–748.
[11] X. Zeng, Y. Zhu, H. Lu, Carcinogenesis 22 (2001) 215–219.
[12] R. Pochampally, C. Li, W. Lu, L. Chen, R. Luftig, J. Lin, J. Chen, Biochem. Biophys. Res. Commun. 279 (2000) 1001–1010.
[13] C.D. Pozniak, S. Radinovic, A. Yang, F. McKeon, D.R. Kaplan, F.D. Miller, Science 289 (2000) 304–306.
[14] V. De Laurenzi, A. Costanzo, D. Barcaroli, A. Terrinoni, M. Falco, M. Annicchiarico-Petruzzelli, M. Levrero, G. Melino, J. Exp. Med. 188 (1998) 1763–1768.
[15] A.I. Zaika, S. Kovalev, N.D. Marchenko, U.M. Moll, Cancer Res. 59 (1999) 3257–3263.
[16] S.W. Chi, A. Ayed, C.H. Arrowsmith, EMBO J. 18 (1999) 4438–4445.
[17] W.K. Wang, M. Bycroft, N.W. Foster, A.M. Buckle, A.R. Fersht, Y.W. Chen, Acta Crystallogr. D Biol. Crystallogr. 57 (2001) 545–551.
[18] K. Hibi, B. Trink, M. Patturajan, W.H. Westra, O.L. Caballero, D.E. Hill, E.A. Ratovitski, J.J. Jen, D. Sidransky, Proc. Natl. Acad. Sci. USA 97 (2000) 5462–5467.
[19] A. Yang, R. Schweitzer, D. Sun, M. Kaghad, N. Walker, R.T. Bronson, C. Tabin, A. Sharpe, D. Caput, C. Crum, F. McKeon, Nature 398 (1999) 714–718.
[20] A.A. Mills, B. Zheng, X.J. Wang, H. Vogel, D.R. Roop, A. Bradley, Nature 398 (1999) 708–713.
[21] J. Celli, P. Duijf, B.C. Hamel, M. Bamshad, B. Kramer, A.P. Smits, R. Newbury-Ecob, R.C. Hennekam, G. Van Buggenhout, A. van Haeringen, C.G. Woods, A.J. van Essen, R. de Waal, G. Vriend, D.A. Haber, A. Yang, F. McKeon, H.G. Brunner, H. van Bokhoven, Cell 99 (1999) 143–153.
[22] G. Pellegrini, E. Dellambra, O. Golisano, E. Martinelli, I. Fantozzi, S. Bondanza, D. Ponzin, F. McKeon, M. De Luca, Proc. Natl. Acad. Sci. USA 98 (2001) 3156–3161.
[23] R. Parsa, A. Yang, F. McKeon, H. Green, J. Invest. Dermatol. 113 (1999) 1099–1105.
[24] K. Nylander, P.J. Coates, P.A. Hall, Int. J. Cancer 87 (2000) 368–372.
[25] V. De Laurenzi, G. Raschella, D. Barcaroli, M. Annicchiarico-Petruzzelli, M. Ranalli, M.V. Catani, B. Tanno, A. Costanzo, M. Levrero, G. Melino, J. Biol. Chem. 275 (2000) 15226–15231.
[26] S. Kovalev, N. Marchenko, S. Swendeman, M. LaQuaglia, U.M. Moll, Cell Growth Differ. 9 (1998) 897–903.
[27] M.P. Tschan, T.J. Grob, U.R. Peters, V.D. Laurenzi, B. Huegli, K. Kreuzer, C.A. Schmidt, G. Melino, M.F. Fe, A. Tobler, J.F. Cajot, Biochem. Biophys. Res. Commun. 277 (2000) 62–65.
[28] V. De Laurenzi, A. Rossi, A. Terrinoni, D. Barcaroli, M.

Levrero, A. Costanzo, R.A. Knight, P. Guerrieri, G. Melino, Biochem. Biophys. Res. Commun. 273 (2000) 342–346.
[29] R. Montes de Oca Luna, D.S. Wagner, G. Lozano, Nature 378 (1995) 203–206.
[30] S.N. Jones, A.E. Roe, L.A. Donehower, A. Bradley, Nature 378 (1995) 206–208.
[31] S. Ikawa, A. Nakagawara, Y. Ikawa, Cell Death Differ. 6 (1999) 1154–1161.
[32] C.A. Jost, M.C. Marin, W.G. Kaelin Jr., Nature 389 (1997) 191–194.
[33] C.J. DiComo, C. Gaiddon, C. Prives, Mol. Cell. Biol. 19 (1999) 1438–1449.
[34] J. Zhu, J. Jiang, W. Zhou, X. Chen, Cancer Res. 58 (1998) 5061–5065.
[35] C.W. Lee, N.B. La Thangue, Oncogene 18 (1999) 4171–4181.
[36] K. Nakano, E. Balint, M. Ashcroft, K.H. Vousden, Oncogene 19 (2000) 4283–4289.
[37] W.T. Steegenga, A. Shvarts, N. Riteco, J.L. Bos, A.G. Jochemsen, Mol. Cell. Biol. 19 (1999) 3885–3894.
[38] X. Zheng, X. Chen, FEBS Lett. 489 (2001) 4–7.
[39] B. Salimath, D. Marme, G. Finkenzeller, Oncogene 19 (2000) 3470–3476.
[40] S. Ishida, T. Yamashita, U. Nakaya, T. Tokino, Jpn. J. Cancer Res. 91 (2000) 174–180.
[41] X. Zeng, X. Li, A. Miller, Z. Yuan, W. Yuan, R.P. Kwok, R. Goodman, H. Lu, Mol. Cell. Biol. 20 (2000) 1299–1310.
[42] X. Zeng, H. Lee, Q. Zhang, H. Lu, J. Biol. Chem. 276 (2001) 48–52.
[43] J. Gu, D. Chen, J. Rosenblum, R.M. Rubin, Z.M. Yuan, Mol. Cell. Biol. 20 (2000) 1243–1253.
[44] E. Balint, S. Bates, K.H. Vousden, Oncogene 18 (1999) 3923–3929.
[45] M.C. Marin, C.A. Jost, M.S. Irwin, J.A. DeCaprio, D. Caput, W.C. Kaelin Jr., Mol. Cell. Biol. 18 (1998) 6316–6324.
[46] X.Q. Wang, W.M. Ongkeko, A.W. Lau, K.M. Leung, R.Y. Poon, Cancer Res. 61 (2001) 1598–1603.
[47] M. Dobbelstein, S. Wienzek, C. Konig, J. Roth, Oncogene 18 (1999) 2101–2106.
[48] X. Zeng, L. Chen, C.A. Jost, R. Maya, D. Keller, X. Wang, W.G. Kaelin, Mol. Cell. Biol. 19 (1999) 3257–3266.
[49] E.A. Ratovitski, M. Patturajan, K. Hibi, B. Trink, K. Yamaguchi, D. Sidransky, Proc. Natl. Acad. Sci. USA 98 (2001) 1817–1822.
[50] K.M. Liefer, M.I. Koster, X.J. Wang, A. Yang, F. McKeon, D.R. Roop, Cancer Res. 60 (2000) 4016–4020.
[51] E. Appella, C.W. Anderson, Eur. J. Biochem. 268 (2001) 2764–2772.
[52] Z.M. Yuan, H. Shioya, T. Ishiko, X. Sun, J. Gu, Y.Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum, D. Kufe, Nature 399 (1999) 814–817.
[53] R. Agami, G. Blandino, M. Oren, Y. Shaul, Nature 399 (1999) 809–813.
[54] A. Minty, X. Dumont, M. Kaghad, D. Caput, J. Biol. Chem. 275 (2000) 36316–36323.
[55] H. Tsao, X. Zhang, P. Majewski, F.G. Haluska, Cancer Res. 59 (1999) 172–174.
[56] S. Nomoto, N. Haruki, M. Kondo, H. Konishi, T. Takahashi, Cancer Res. 58 (1998) 1380–1383.
[57] M. Mai, C. Qian, A. Yokomizo, D.J. Tindall, D. Bostwick, C. Polychronakos, D.I. Smith, W. Liu, Oncogene 17 (1998) 1739–17341.
[58] M. Mai, A. Yokomizo, C. Qian, P. Yang, D.J. Tindall, D.I. Smith, W. Liu, Cancer Res. 58 (1998) 2347–2349.
[59] Y.C. Cai, G.Y. Yang, Y. Nie, L.D. Wang, X. Zhao, Y.L. Song, D.N. Seril, J. Liao, E.P. Xing, C.S. Yang, Carcinogenesis 21 (2000) 683–689.
[60] M. Reichelt, K.D. Zang, M. Seifert, C. Welter, T. Ruffing, Arch. Virol. 144 (1999) 621–626.
[61] S. Wienzek, J. Roth, M. Dobbelstein, J. Virol. 74 (2000) 193–202.
[62] J.S. Park, E.J. Kim, J.Y. Lee, H.S. Sin, S.E. Namkoong, S.J. Um, Int. J. Cancer 91 (2001) 822–827.
[63] N.S. Prabhu, K. Somasundaram, K. Satyamoorthy, M. Herlyn, W.S. El-Deiry, Int. J. Oncol. 13 (1998) 5–9.
[64] F. Higashino, J.M. Pipas, T. Shenk, Proc. Natl. Acad. Sci. USA 95 (1998) 15683–15687.
[65] A. Kaida, Y. Ariumi, Y. Ueda, J.Y. Lin, M. Hijikata, S. Ikawa, K. Shimotohno, Oncogene 19 (2000) 827–830.
[66] J. Roth, M. Dobbelstein, J. Gen. Virol. 80 (1999) 3251–3255.
[67] Y. Tokuchi, T. Hashimoto, Y. Kobayashi, M. Hayashi, K. Nishida, S. Hayashi, K. Imai, K. Nakachi, Y. Ishikawa, K. Nakagawa, Y. Kawakami, E. Tsuchiya, Br. J. Cancer 80 (1999) 1623–1629.
[68] M.J. Kang, B.J. Park, D.S. Byun, J.L. Park, H.J. Kim, J.H. Park, S.G. Chi, Clin. Cancer Res. 6 (2000) 1767–1771.
[69] M. Sunahara, S. Ichimiya, Y. Nimura, N. Takada, S. Sakiyama, Y. Sato, S. Todo, W. Adachi, J. Amano, A. Nakagawara, Int. J. Oncol. 13 (1998) 319–323.
[70] S.G. Chi, S.G. Chang, S.J. Lee, C.H. Lee, J.L. Kim, J.H. Park, Cancer Res. 59 (1999) 2791–2793.
[71] A. Yokomizo, M. Mai, D.J. Tindall, L. Cheng, D.G. Bostwick, S. Naito, D.I. Smith, W. Liu, Oncogene 18 (1999) 1629–1633.
[72] S.W. Ng, G.K. Yiu, Y. Liu, L.W. Huang, M. Palnati, S.H. Jun, R.S. Berkowitz, S.C. Mok, Oncogene 19 (2000) 1885–1890.
[73] C.L. Chen, S.M. Ip, D. Cheng, L.C. Wong, H.Y. Ngan, Clin. Cancer Res. 6 (2000) 3910–3915.
[74] D. Zwahlen, M.P. Tschan, T.J. Grob, U.R. Peters, D. Fink, W. Haenggi, H.J. Altermatt, J.F. Cajot, A. Tobler, M.F. Fey, S. Aebi, Int. J. Cancer 88 (2000) 66–70.
[75] H. Loiseau, J. Arsaut, J. Demotes-Mainard, Neurosci. Lett. 263 (1999) 173–176.
[76] N.I. Herath, M.C. Kew, V.L. Whitehall, M.D. Walsh, J.R. Jass, K.K. Khanna, J. Young, L.W. Powell, B.A. Leggett, G.A. Macdonald, Hepatology 31 (2000) 601–605.
[77] A. Tannapfel, M. Wasner, K. Krause, F. Geissler, A. Katalinic, J. Hauss, J. Mossner, K. Engeland, C. Wittekind, J. Natl. Cancer Inst. 9 (1999) 1154–1158.
[78] A. Tannapfel, K. Engeland, L. Weinans, A. Katalinic, J.

Hauss, J. Mossner, C. Wittekind, Br. J. Cancer 80 (1999) 1069–1074.
[79] U.R. Peters, M.P. Tschan, K.A. Kreuzer, G. Baskaynak, U. Lass, A. Tobler, M.F. Fey, C.A. Schmidt, Cancer Res. 59 (1999) 4233–4236.
[80] P.G. Corn, S.J. Kuerbitz, M.M. van Noesel, M. Esteller, N. Compitello, S.B. Baylin, J.G. Herman, Cancer Res. 59 (1999) 3352–3356.
[81] S. Kawano, C.W. Miller, A.F. Gombart, C.R. Bartram, Y. Matsuo, H. Asou, A. Sakashita, J. Said, E. Tatsumi, H.P. Koeffler, Blood 94 (1999) 1113–1120.
[82] I. Fillippovich, N. Sorokina, M. Gatei, Y. Haupt, K. Hobson, E. Moallem, K. Spring, M. Mould, M.A. McGuckin, M.F. Lavin, K.K. Khanna, Oncogene 20 (2001) 514–522.
[83] B.J. Park, S.J. Lee, J.I. Kim, S.J. Lee, C.H. Lee, S.G. Chang, J.H. Park, S.G. Chi, Cancer Res. 60 (2000) 3370–3374.
[84] T. Crook, J.M. Nicholl, L. Brooks, J. O'Nions, M.J. Allday, Oncogene 10 (2000) 3439–3444.
[85] A. Zaika, M. Irwin, C. Sansome, U.M. Moll, J. Biol. Chem. 276 (2001) 11310–11316.
[86] T. Stiewe, B.M. Putzer, Nat. Genet. 26 (2000) 464–469.
[87] N.A. Lissy, P.K. Davis, M. Irwin, W.G. Kaelin, S.F. Dowdy, Nature 407 (2000) 642–645.
[88] M. Irwin, M.C. Marin, A.C. Phillips, R.S. Seelan, D.I. Smith, W. Liu, E.R. Flores, K.Y. Tsai, T. Jacks, K.H. Vousden, W.G. Kaelin Jr., Nature 407 (2000) 645–648.
[89] L. Yamasaki, T. Jacks, R. Bronson, E. Goillot, E. Harlow, N.J. Dyson, Cell 85 (1996) 537–548.
[90] S.J. Field, F.Y. Tsai, F. Kuo, A.M. Zubiaga, W.G. Kaelin, Cell 85 (1996) 549–561.
[91] M. Liu, T. Taketani, R. Li, J. Takita, T. Taki, H.W. Yang, H. Kawaguchi, K. Ida, Y. Matsuo, Y. Hayashi, Leuk. Res. 25 (2001) 441–447.
[92] P. Scaruffi, I. Casciano, L. Masiero, G. Basso, M. Romani, G.P. Tonini, Leukemia 14 (2000) 518–519.
[93] M. Herranz, J. Santos, E. Salido, J. Fernandez-Piqueras, M. Serrano, Cancer Res. 59 (1999) 2068–2071.
[94] L. Fang, S.W. Lee, S.A. Aaronson, J. Cell Biol. 147 (1999) 823–830.
[95] J.G. Gong, A. Costanzo, H.Q. Yang, G. Melino, W.G. Kaelin Jr., M. Levrero, J.Y. Wang, Nature 399 (1999) 806–809.
[96] I. Katoh, K.I. Aisaki, S.I. Kurata, S. Ikawa, Y. Ikawa, Oncogene 19 (2000) 3126–3130.
[97] M.C. Marin, C.A. Jos, L.A. Brooks, M.S. Irwin, J. O'Nions, J.A. Tidy, N. James, J.M. McGregor, C.A. Harwood, I.G. Yulug, K.H. Vousden, M.J. Allday, B. Gusterson, S. Ikawa, P.W. Hinds, T. Crook, W.G. Kaelin Jr., Nat. Genet. 25 (2000) 47–54.
[98] S. Strano, E. Munarriz, M. Rossi, B. Cristofanelli, Y. Shaul, L. Castagnoli, A.J. Levine, A. Sacchi, G. Cesareni, M. Oren, G. Blandino, J. Biol. Chem. 275 (2000) 29503–29512.
[99] T.S. Davison, C. Vagner, M. Kaghad, A. Ayed, D. Caput, C.H. Arrowsmith, J. Biol. Chem. 274 (1999) 18709–18714.
[100] C. Gaiddon, M. Lokshin, J. Ahn, T. Zhang, C. Prives, Mol. Cell. Biol. 21 (2001) 1874–1887.
[101] S.E. Kern, J.A. Pietenpol, S. Thiagalingam, A. Seymour, K.W. Kinzler, B. Vogelstein, Science 256 (1992) 827–830.
[102] T. Unger, M.M. Nau, S. Segal, J.D. Minna, EMBO J. 11 (1992) 1383–1390.
[103] D. Dittmer, S. Pati, G. Zambetti, S. Chu, A.K. Teresky, M. Moore, C. Finlay, A.J. Levine, Nat. Genet. 4 (1993) 42–46.
[104] G. Shaulsky, N. Goldfinger, V. Rotter, Cancer Res. 51 (1991) 5232–5237.
[105] O. Halevy, D. Michalovitz, M. Oren, Science 250 (1990) 113–116.
[106] F. Vikhanskaya, M. D'Incalci, M. Broggini, Nucleic Acids Res. 28 (2000) 513–519.
[107] Y. Ueda, M. Hijikata, S. Takagi, T. Chiba, K. Shimotohno, Oncogene 18 (1999) 4993–4998.

ΔNp73, A Dominant-Negative Inhibitor of Wild-type p53 and TAp73, Is Up-regulated in Human Tumors

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Abstract

p73 has significant homology to p53. However, tumor-associated up-regulation of p73 and genetic data from human tumors and p73-deficient mice exclude a classical Knudson-type tumor suppressor role. We report that the human TP73 gene generates an NH_2 terminally truncated isoform. ΔNp73 derives from an alternative promoter in intron 3 and lacks the transactivation domain of full-length TAp73. ΔNp73 is frequently overexpressed in a variety of human cancers, but not in normal tissues. ΔNp73 acts as a potent transdominant inhibitor of wild-type p53 and transactivation-competent TAp73. ΔNp73 efficiently counteracts transactivation function, apoptosis, and growth suppression mediated by wild-type p53 and TAp73, and confers drug resistance to wild-type p53 harboring tumor cells. Conversely, down-regulation of endogenous ΔNp73 levels by antisense methods alleviates its suppressive action and enhances p53- and TAp73-mediated apoptosis. ΔNp73 is complexed with wild-type p53, as demonstrated by coimmunoprecipitation from cultured cells and primary tumors. Thus, ΔNp73 mediates a novel inactivation mechanism of p53 and TAp73 via a dominant-negative family network. Deregulated expression of ΔNp73 can bestow oncogenic activity upon the TP73 gene by functionally inactivating the suppressor action of p53 and TAp73. This trait might be selected for in human cancers. [Q2]

Key words: p73 • ΔNp73 • Ex2Del p73 • apoptosis • deregulation in tumor

Introduction

The p53 family member p73 has significant homology to the p53 tumor suppressor. Human full-length p73 (TAp73) shares 63% amino acid identity with the DNA-binding region of TP53 including conservation of all DNA contact residues, as well as 38 and 29% identity with the tetramerization domain and transactivation domain, respectively (1). TAp73 also shows functional homology to p53. Ectopically overexpressed TAp73α and TAp73β (two COOH-terminal splice variants) largely mimic p53 activities, including the induction of apoptosis, cell cycle arrest, and the transactivation of an overlapping set of target genes (2, 3). Moreover, endogenous TAp73 is able to integrate death stimuli from three different pathways: cellular and viral oncogenes, some forms of DNA damage, and T cell receptor hyperactivation. We and others have shown that deregulation of the oncogenes E2F1, cMyc, and E1A induces apoptosis and growth suppression in tumor cells in a p53-independent manner by transcriptionally inducing and activating endogenous TAp73 proteins (4–7). Moreover, during E2F1-mediated apoptosis in primary mouse embryo fibroblasts (MEFs),* a supra-additive cooperation exists between wild-type p53 and TAp73 (5). Although wild-type MEFs show 77% apoptosis after forced E2F1 expression, $p53^{-/-}$ MEFs (containing TAp73) and $p73^{-/-}$ MEFs (containing p53) alone show reduced killing ability of only 12 and 15%, respectively. The excessively weakened killing ability of $p73^{-/-}$ MEFs, despite the presence of wild-type p53, is consistent with an important synergistic signal emanating from TAp73 that cooperates with p53 to induce oncogene-triggered death. Because oncogene deregulation of E2F1 is one of the most common genetic alterations in human tumors, this finding provides a possible physiologic cause for TAp73 overexpression in tumors. Furthermore,

( ) brackets

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*Abbreviations used in this paper: EMSA, mobility shift assay; GAPDH, glyceraldehyde3-phosphate dehydrogenase; GFP, green fluorescent protein; MEF, mouse embryo fibroblast; TUNEL, Tdt-mediated dUTP-X nick-end labeling; UTR, untranslated region.

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TAp73 is activated to mediate apoptosis by a restricted spectrum of DNA damage. Endogenous TAp73 is activated in response to cisplatin and γ-IR in a pathway that depends on the nonreceptor tyrosine kinase c-abl (8–10). Moreover, doxorubicin stabilizes TAp73 protein by acetylation (11). Conversely, cells deficient in c-abl do not upregulate their p73 and are resistant to killing by cisplatin. On the other hand, TAp73 is not activated by UV, actinomycin D, and mitomycin C, all of which activate p53 (1, 8). Lastly, peripheral T cells undergo apoptosis after hyperstimulation of their T cell receptors. This cell fate is mediated via the E2F1–TAp73 pathway (6). Consistent with this notion, E2F1 null mice exhibit a marked disruption of lymphatic homeostasis with increased T cells and splenomegaly (12, 13). Thus, TAp73 might function independently but synergistically with p53 in a tumor surveillance pathway in vivo.

However, despite this strong functional homology, data from human tumors and p73-deficient mice argue against a classical Knudson-type tumor suppressor role for the TP73 gene. TP73-deficient mice lack a spontaneous tumor phenotype (14) and inactivating mutations in human tumors are extremely rare (>900 tumors analyzed to date; for review see reference 15). Moreover, although all normal human tissues studied express very low levels of p73, multiple primary tumor types and tumor cell lines overexpress wild-type p73, including cancers of the breast, lung, esophagus, stomach, colon, bladder, ovary, liver, bile ducts, ependymal lining, myelogenous leukemia, and neuroblastoma (15). It is important to point out that to date, most studies identifying p73 overexpression in primary human tumors have examined total levels of p73, with only a few exceptions that specifically measured TAp73 (16, 17). Importantly, in the mouse, an N terminally truncated ΔNp73 protein has recently been found, generated from an alternative promoter in intron 3 and lacking a transactivation domain (18). ΔNp73 is the predominant form in the developing mouse brain and is the only form of p73 in the neonatal brain and sympathetic ganglia (14, 18). Mouse ΔNp73 plays an essential antiapoptotic role during developmental p53-driven neuronal death in vivo by acting as a dominant-negative inhibitor of p53 (18). Functional studies have shown that ΔNp73 is required to counteract p53-mediated neuronal death during normal "sculpting" of the developing mouse neuronal system (18). In primary neuronal cultures, withdrawal of the obligate survival factor NGF **[Q3]** leads to p53 induction with p53-dependent cell death but a concomitant decrease of ΔNp73. Importantly, sympathetic neurons are rescued from cell death after NGF **[Q3]** withdrawal or recombinant adenoviral p53 infection when ΔNp73 levels are maintained by viral delivery (18). Given the existence of this powerful transdominant p53 inhibitor in the mouse, the possibility arose that this isoform might in part be responsible for the overexpression seen in human tumors and, in fact, be the crucial component. Therefore, we sought the human counterpart of ΔNp73, determined whether human tumors express it and determined its potential role in cancer.

Materials and Methods

Tumor Samples and Cell Lines. Primary tumors and normal tissues were collected at University Hospital State University of New York, Stony Brook in compliance with and approved by the Institutional Review Board. Freshly harvested tumors (minimum 60% tumor cells) and normal tissues were immediately snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. The human breast cancer cell line MDA 231, the p53 null lines H1299 and SaOs2, and the wild-type p53 lines U2OS, RKO, and HeLa cells were maintained in DMEM/10% fetal calf serum.

Semiquantitative RT-PCR Assay. Total RNA was extracted from tissues as previously described (17). 1 μg total RNA, 10 pmoles each of a radiolabeled isoform-specific upstream primer, and a common TP73 exon 4 reverse primer were used in a 10-μl reaction (Titan Kit; Roche) for 25 cycles to ensure linearity of the assay (unpublished data) and subjected to phosphoimage analysis. Primer sequences were 5′-TGC TGT ACG TCG GTG ACC-3′ (sense ΔNp73), 5′-CGA CGG CTG CAG AGC GAG-3′ (sense TAp73), and 5′-TCG AAG GTG GAG CTG GGT TG-3′ (antisense for both). ΔNp73 and TAp73 amplicon sizes were 175 and 365 bp, respectively. For Ex2Del p73, primers were 5′-GCG CCA GGC CAG CCG GGA CGG AC-3′ (sense) and 5′-CGC GGC TGC TCA TCT GGT CCA TGG TGC-3′ (antisense), which yielded a 320-bp band. Band intensities were normalized to their respective glyceraldehyde3-phosphate dehydrogenase (GAPDH) values. In matched samples, the up-regulation in tumors was calculated by dividing normalized $\Delta Np73^{Tumor}$ by $\Delta Np73^{Normal}$, $TAp73^{Tumor}$ by $TAp73^{Normal}$, or Ex2Del $p73^{Tumor}$ by Ex2Del $p73^{Normal}$. To calculate the preferential up-regulation of ΔNp73 in tumors, the formula $\Delta Np73^{Tumor}/\Delta Np73^{Normal}$ divided by $TAp73^{Tumor}/TAp73^{Normal}$ was used. The analogous formula was used for Ex2Del p73. Some samples were independently repeated and yielded highly reproducible results. Genomic sequencing of p53 exons 5–9 was performed with the Amplimer Panel (CLONTECH Laboratories, Inc.). Immunocytochemical staining for p53 was done on fixed tissue sections from the same tumor mass.

Luciferase Assays A pcDNA3-based expression plasmid for ΔNp73α was generated with an NH_2-terminal Flag tag. pcDNA3-Fp53 expressing human Flag-tagged wild-type p53, pcDNA3-p73α and pcDNA3-p73β expressing hemagglutinin-tagged human TAp73α and TAp73β (provided by G. Melino, **[Q4]**), murine stem cell virus expressing a tetramerization domain mutant of ΔNp73α called ΔNp73L322P (corresponding to L371P in TAp73α), and the p53/p73-specific reporter PG13-Luc were previously described (4). PG13 is a reporter plasmid containing 13 tandem repeats of the p53 consensus DNA binding site. H1299 and SaOs2 cells were transfected by Fugene (Roche). Luciferase activity was normalized for renilla luciferase activity (Promega).

Western Blot and Coimmunoprecipitation Analysis. Hela and H1299 cells were transfected with expression plasmids for [redacted] for wild-type p53 or TAp73β with either 1.5 μg ΔNp73α or empty vector (see Fig. 3 C). The indicated molar ratios were used and green fluorescent protein (GFP) was cotransfected in all cases (see Fig. 3 B). Total cell lysates were prepared 24 h later and subjected to immunoblot analysis. Gel loading was normalized for equal vimentin or GFP levels (20 μg per lane). Antibodies to p73 were monoclonal ER15 (recognizes amino acids 380–495 of human p73α isoforms and detects TA and ΔN forms; Oncogene Research Products), polyclonal anti-p73 (raised against the COOH-terminal; Chemicon), and the polyclonal anti-ΔNp73 (raised in

rabbit against the exon 3′ peptide LYVGDPARHLATA and immunopurified, and does not cross react with p53 or any TAp73 isoform). Antibodies against p53 (DO-1 and PAb 421), HDM2 (IF2), p21Waf1, and 14-3-3σ were from Oncogene Research Products and Flag antibody (M2) was from Sigma-Aldrich. For the normalization of protein loading, blots were reprobed with α-vimentin (BioGenex) or GFP (CLONTECH Laboratories, Inc., see Fig. 3 B). For coimmunoprecipitations (see Fig. 5, A–C), SaOs2 and U2OS cells were transfected with 2.4 μg of the indicated plasmids in single transfections, or 0.6 μg p53 plasmid plus 1.8 μg ΔNp73α in cotransfections. 24 h later, 600 μg lysates were subjected to immunoprecipitation with 1 μg of the indicated antibodies and analyzed by Western blot as previously described (19).

For the immunoprecipitation of ΔNp73 from tumors and normal tissues (see Fig. 2 D), frozen tissue was mechanically pulverized under liquid N2, resuspended in 1.5 ml TENN buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, pH 8.0) containing a protease inhibitor cocktail (Roche), and homogenized using an electric homogenizer followed by sonication for 2 min. The lysates were centrifuged twice at 14,000 rpm for 15 min. 2 mg total protein in 500 μl TENN was precleared four times using 70 μl each time of a 1:1 mixture of protein A and G slurry (GIBCO BRL). The last supernatant was incubated with 1 μg ER15 antibody for 30 min at 4°C, followed by the addition of 40 μl slurry of Prot G beads. The mixture was then rotated overnight at 4°C. For controls, lysates were immunoprecipitated with monoclonal GC15 specific for p73β (Oncogene Research Products) or Flag antibody (M2). Pelleted beads were washed four times in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% NaDeoxycholate, pH 7.4), boiled in sample buffer for 3 min, and loaded onto an SDS-PAGE gel. Membranes were immunoblotted with polyclonal anti-ΔNp73 (1:20) and bands were verified by reblotting with ER15 (1:200). For coimmunoprecipitation of a ΔNp73–p53 complex from tissues (see Fig. 5 D), 2 mg total protein in 500 μl TENN was precleared as described above and incubated with a 1:1 mixture (40 μl slurry) of agarose beads covalently coupled to the monoclonal p53 antibodies 1801 or DO-1 (Santa Cruz Biotechnology, Inc.). For controls, 40 μl Prot G agarose slurry was used. Membranes were immunoblotted with polyclonal anti-ΔNp73 and bands were verified by reblotting with ER15.

Apoptosis Assay. Hela and SaOs2 cells were seeded into 8-well chamber slides and cotransfected with 300 ng of the indicated pcDNA3-based expression plasmids per well using Fugene (see Fig. 4 A). Control wells (vector alone) received 600 ng. After 16 or 24 h, cells were stained with Annexin V or Tdt-mediated dUTP-X nick-end labeling (TUNEL), respectively, according to the manufacturer's instructions (Roche). Expression was determined by immunofluorescence in duplicate wells. Transfection efficiency was reproducibly ~30% of cells, similar among all constructs and evenly distributed throughout the wells. Annexin V or TUNEL positive cells (494 fields at 40×) and plasmid-expressing cells (15 random fields, >500 cells) were counted and the percentage of apoptosis in transfected cells was determined after correction for background with vector alone.

RKO cells were seeded into 8-well slides and transfected with either vector, the irrelevant control plasmid cRel, or with ΔNp73α using Lipofectamine Plus (GIBCO BRL; see Fig. 4 D). LcRel is a transcriptionally and apoptotically inactive Flag-tagged, truncated version of the transcription factor cRel, fused to a mitochondrial targeting sequence (19) and cloned into pcDNA3. 5 h after transfection, cells were either stressed with 5 μM camptothecin or left untreated overnight before fixation with paraformaldehyde. Wells were processed for TUNEL staining followed by immunofluorescence staining with polyclonal anti-ΔNp73 (1:20) and donkey anti–rabbit IgG (H+L)-rhodamine conjugate (Jackson ImmunoResearch Laboratories). Apoptosis was quantitated as the percentage of ΔNp73α-expressing cells and compared with vector-transfected cells.

Colony Suppression Assay. SaOs2 cells in 60-mm plates were transfected by Fugene with 1.5 μg each of the indicated expression plasmids. Vector-only plates received 3 μg plasmid. 24 h later, cells were transferred to 100-mm dishes and placed under G418 selection (500 μg/ml) for 21 d, fixed, stained with crystal violet (20), and photographed. All foci were counted.

Mobility Shift Assays (EMSAs). H1299 and U2OS cells were transfected with plasmids encoding wild-type p53, ΔNp73α, or a combination of the two. 24 h later, nuclear extracts were prepared by Dounce homogenization as previously described (21). As probes, we used double stranded oligonucleotides of the p53 consensus DNA binding sequences: 5′-GGGCATGTC-CGGGCATGTCC-3′ (called p53CON) or 5′-CCTGCCTG-GACTTGCCTGGCCTGCCTGGACTTGCCTGG-3′.
EMSA was performed with 1 ng 32 P–labeled probe and 10 μg nuclear extract in binding buffer (40 mM NaCl, 10 mM morpholino propane sulfonic acid, pH 7.0, 0.1% NP-40, 1 mM EDTA, 2.5% glycerol, 0.5 μg poly[dI-dC]) for 30 min at room temperature. Samples were loaded onto a native 6% polyacrylamide gel and electrophoresed at 4°C and 200 V for 4 h. Where indicated, 0.5 μg p53-specific monoclonal antibody PAb 421 was included to produce a supershift. Specific competition experiments included unlabeled p53CON in 50-fold molar excess of radiolabeled probe. Nonspecific competition consisted of 50-fold molar excess of the scrambled p53CON 5′-GGGAATTTC-CGGGAATTTCC-3′ or the mutant sequence CCTTAATG-GACTTTAATGGCCTTAATGGACTTTAATGG (mutated nucleotides are underlined).

Antisense Suppression of ΔNp73. Wild-type p53-harboring RKO cells were seeded into 8-well slides and transfected with 360 ng wild-type p53 expression plasmid with 200 nM of either ΔNp73 antisense oligonucleotide directed against exon 3′ with extension into the adjacent 5′ untranslated region (UTR) (antisense, 5′-A*C*C*G*ACGTACAGC*A*T*G*G-3′; stars indicate the phosphorothioate-modified bases), or ΔNp73 sense oligonucleotide (HPLC-purified) (sense, 5′-C*C*A*T*GCTG-TACGT*C*G*G*T-3′) (Operon Inc.) using Lipofectamine Plus or Oligofectamine (GIBCO BRL) according to the manufacturer's instructions. Vector alone was also used. 20 h after transfection, cells were subjected to immunoblotting or TUNEL staining. Apoptosis was quantitated using a Nikon E800 microscope equipped with a Bio-Rad confocal laser scanning system. For each sample, the total fluorescence of five random fields per duplicate well was acquired with a 20× lens using identical acquisition and photomultiplier settings. Data were processed to calculate the integral optical density using the Laser Pix software package (Bio-Rad Laboratories). In other experiments, RKO cells were transfected with 200 nM each of the antisense and sense oligonucleotides listed above. After 8 h, cells were DNA damaged by adding 1 μM camptothecin for an additional 16 h before TUNEL staining. Apoptosis was determined as described above.

Results

The Human TP73 Gene Can Produce ΔNp73. Mouse ΔNp73 differs from TAp73 by a novel exon 3′, which re-

should read: ... or ΔNp73 sense oligonucleotide (sense, 5′- .....) (both HPLC-purified, Operon Inc.)

places the first 3 exons and is spliced in frame to exon 4 of the TP73 gene (18). To clone the human homologue of mouse ΔNp73, we performed a GenBank search using mouse exon 3′. By sequence alignment of a human genomic PAC [Q5] clone containing TP73 (sequence data are available from Genbank/EMBL/DDBJ under accession no. AL 136528), we identified a region with 77% identity to the 5′ UTR of mouse ΔNp73 mRNA (sequence data are available from Genbank/EMBL/DDBJ under accession no. Y 19235). This allowed us to predict the human exon 3′ and design isoform-specific primers for human ΔNp73. Full-length ΔNp73α cDNA, spanning exons 3′-14 including 103 bases of 3′ UTR, was cloned by RT-PCR from total RNA of human placenta and MDA 231 breast cancer cells, and sequence was confirmed (Fig. 1 A). To confirm that ΔNp73 derives from a separate promoter upstream of exon 3′, we performed 5′ rapid amplification of cDNA ends and sequenced the 5′ UTR upstream of exon 3′ (Fig. 1 C). Human exon 3′ encodes 13 unique amino acids with almost complete identity to mouse exon 3′ (12 out of 13 residues are identical; Fig. 1 A). The human ΔN promoter contains the predicted TATA box 25 nt [Q6] upstream of the transcriptional start site, which is located 7.6 kb downstream of exon 3.

Expression of ΔNp73 and Ex2Del p73 in Human Tumors. Unique cDNA primers were designed for amplification of ΔNp73 from tissues by semiquantitative RT-PCR (Fig. 1 B). Then, we determined if expression levels of ΔNp73 were tumor specifically up-regulated using a spectrum of human tumor pairs that were matched with the patients' normal tissues of origin (Table I and Fig. 2 A). They included 35 cancers (cancers of the ovary, endometrium, cervix, vulva, vagina, breast, kidney, and colon) and 2 large benign ovarian tumors (serous cystadenoma and serous cystadenofibroma). Indeed, in 27 of the 37 pairs (73%), ΔNp73 was specifically up-regulated between 2- and 150-fold in the patients' tumors compared with their matched normal tissues (Table I, second column). In a subset of our matched pairs, we also determined the expression of the previously described Ex2Del p73 [Q7]. Ex2Del p73 is another isoform of p73 that lacks most of the transactivation domain, but in contrast to ΔNp73 it is generated from the same promoter as TAp73 by splicing out exon 2. Ex2Del p73 was shown to be up-regulated in some ovarian and vulval cancers and breast cancer cell lines and is a transdominant inhibitor of p53 (22–24). As already seen with ΔNp73, Ex2Del p73 was also specifically up-regulated 2.2–20-fold in 11 of 22 analyzed tumors (50%) compared with their matched normal tissues either alone or concomitant with ΔNp73 (Table I, third column, and Fig. 2 A). Taken together, 30 of 37 tumor pairs (81%) exhibited tumor-specific up-regulation of ΔNp73 and/or Ex2Del p73. On the other hand, because we previously showed that breast cancers can overexpress TAp73 (17), we next used isoform-specific RT-PCR to simultaneously measure ΔNp73 and TAp73. TAp73 was up-regulated in 18 of 37 tumors (49%; Table I, fourth column) compared with its respective normal tissues of origin. However, although tu-

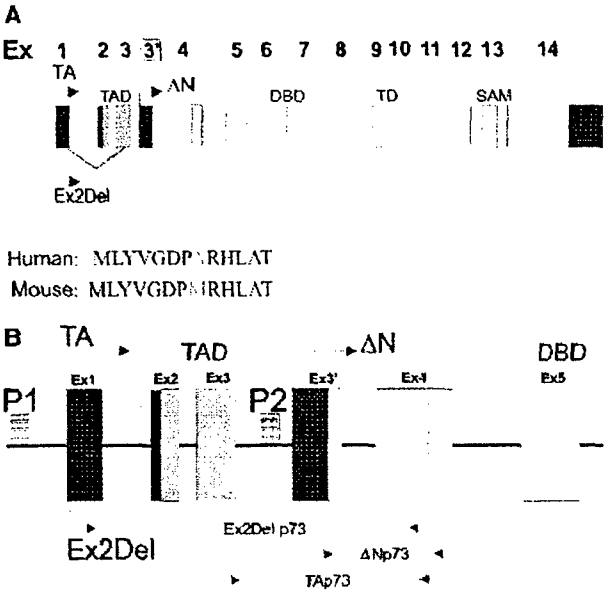


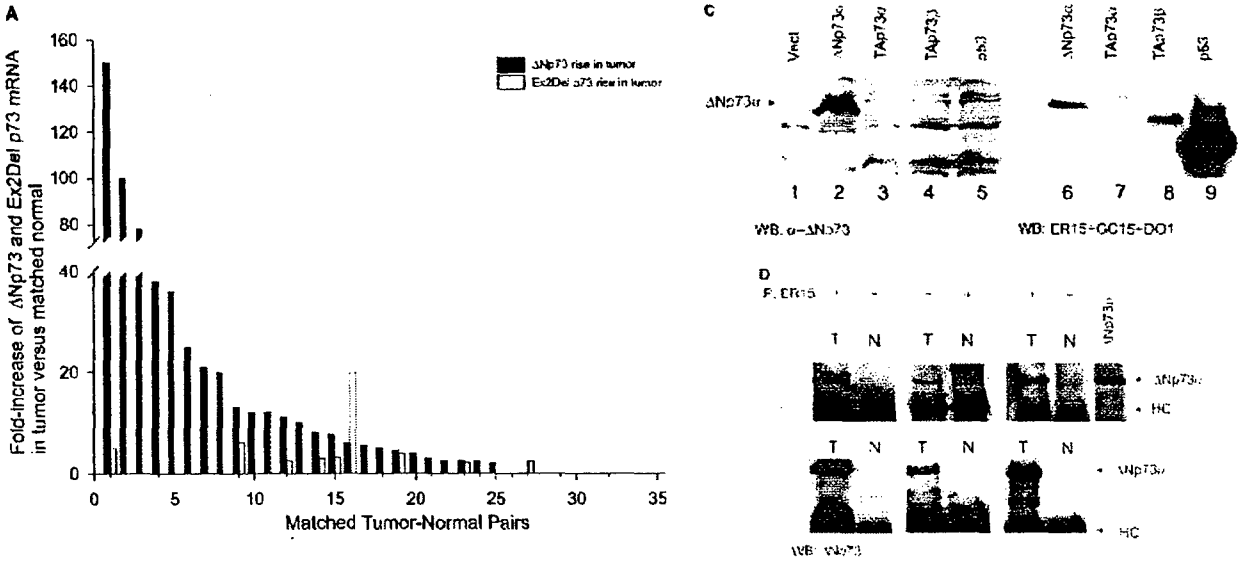
Figure 1. Gene architecture of human TP73. (A) In contrast to TP53, which harbors a single promoter generating a single protein composed of the transactivation domain (TAD), DNA-binding domain (DBD), and tetramerization domain (TD), the TP73 gene is complex and contains two promoters and an additional sterile α motif domain (SAM). The P1 promoter in the 5′ UTR region produces transactivation-competent full-length proteins containing the TA domain (TAp73). The P2 promoter in intron 3 produces TA-deficient protein(s) (ΔNp73) with dominant-negative function toward TAp73 and wild-type p53. ΔNp73 starts with exon 3′, which encodes 13 unique amino acids that are highly conserved between human and mouse. Another NH_2 terminally truncated p73, Ex2Del, also lacks the TA domain but is created by splicing out exon 2 from the P1 transcript (reference 1). The COOH-terminal of TAp73 undergoes additional exon splicing, which generates β–ϕ isoforms. (B) Positions of the P1 and P2 promoters. Positions of the RT-PCR primers are indicated: TAp73 (bottom), ΔNp73 (center), and Ex2Delp73 (top). (C) Sequence of the 5′ UTR region of ΔNp73. The putative TATA box is indicated.

Table I. *Expression of ΔNp73, ExDel p73, and TAp73 in Matched Human Tumors*

No.	Tumor	Increased ΔNp73 expression in tumor vs. normal	Increased Ex2Del expression in tumor vs. normal	Increased TAp73 expression in tumor vs. normal	Preferential up-regulation of ΔNp73 or Ex2Del vs. TAp73 in tumors		p53 status
1	Endometrial Ca	150	5	6.5	23	—	wt, sequence confirmed
2	Endometrial Ca	100	—	7	14.2	—	mut
3	Cervical Ca[a]	78	nd	7	11	nd	wt
4	Vaginal Ca	38	—	—	2,794	—	mut
5	Ovarian Ca	36	nd	3	12	nd	wt, sequence confirmed
6	Cervical Ca	25	nd	155	—	nd	mut Ex 8, R273H CGT→CAT
7	Ovarian Ca	21	nd	4	5	nd	wt, sequence confirmed
8	Ser cystadenofibr	20	—	—	21	—	wt
9	Ovarian Ca	13	6.1	10.5	1.2	—	mut
10	Ovarian Ca	12	—	—	26.6	—	wt
11	Breast Ca	12	nd	—	11	nd	wt, sequence confirmed
12	Endometrial Ca	11	2.5	10	1.1	—	wt
13	Breast Ca[a]	10	nd	—	16	nd	wt, sequence confirmed
14	Endometrial Ca	8	3	32	—	—	wt
15	Vulvar Ca	7.6	3.2	5.3	1.4	—	mut
16	Endometrial CaSar	6	20	—	6	—	mut
17	Ovarian Ca	5.5	—	—	yes*	—	wt
18	Ovarian Ca	5	—	6.6	—	—	mut
19	Ovarian Ca	4.6	4	42	—	—	mut
20	Ovarian Ca	4	nd	10	—	nd	mut Ex 5, C176Y CGC→TGC mut Ex 6, R213STOP CGA→TGA
21	Ovarian Ca[a]	yes*	nd	—	yes*	nd	
22	Ovarian Ca	yes*	nd	yes*	yes*	nd	wt
23	Ser cystadenoma	3	nd	—	8	nd	wt
24	Ovarian Ca	2.6	—	13	—	—	mut
25	Vulvar Ca	2.5	2.2	3.7	—	—	wt
26	Bord Muc Ov Tu	2.4	—	—	2.3	—	wt
27	Ovarian Ca	2	—	3.3	—	—	mut
28	Breast Ca[a]	—	nd	29	—	nd	wt
29	Endometrial Ca	—	2.4	—	—	2	wt
30	Endometrial Ca	—	—	—	—	—	wt
31	Ovarian Ca	—	—	—	—	—	wt
32	Ovarian Ca	—	yes*	—	—	yes*	wt
33	Ovarian Ca[a]	—	nd	—	—	nd	
34	Breast Ca	—	nd	—	—	nd	wt
35	Ovarian Ca	—	nd	—	—	nd	mut
36	Renal Ca	—	nd	—	—	nd	
37	Colon Ca	—	yes*	—	—	yes*	

Numbers reflect fold increase. yes*, cannot be calculated as fold increase because level in normal tissue is undetectable; Ca, carcinoma; ICC, immunocytochemistry for p53 with monoclonal antibody DO-1 (negative suggests wild-type status, positive suggests mutant status); nd, not determined; Ser cystadenofibr, serious cystadenofibroma (a large benign ovarian tumor); Ser cystadenoma, serious cystadenoma (a large benign ovarian tumor); Bord Muc Ov Tu, borderline mucinous ovarian tumor (a large ovarian tumor of low or uncertain malignant potential).

[a]Average of two independent measurements. Values differed by <10%.



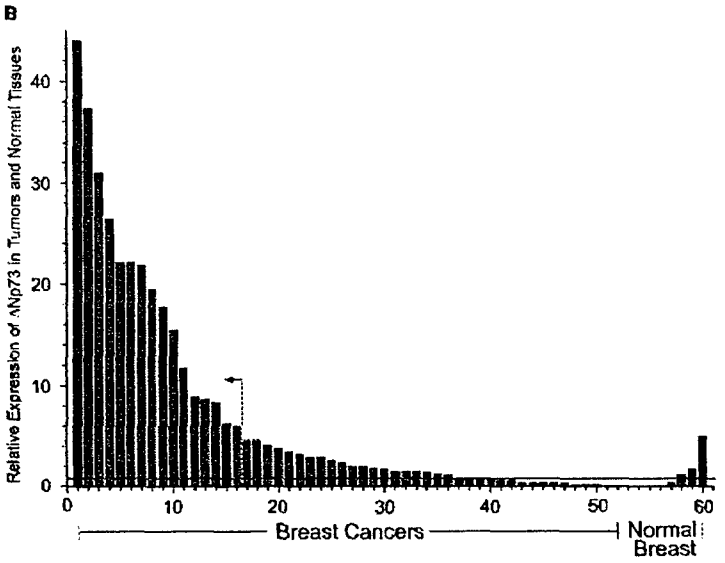


Figure 2. ΔNp73 is frequently overexpressed in a variety of primary human cancers. (A) Tumor-specific up-regulation of ΔNp73 (solid bars) transcripts in 35 tumor pairs, compared with their respective normal tissues of origin. In addition, Ex2Del p73 transcripts (gray bars) were measured in a subset of pairs. Ex2Del p73 is another previously described isoform of p73 that lacks most of the transactivation domain. In contrast to ΔNp73, it is generated from the same promoter as TAp73 by splicing out exon 2. It is also a transdominant inhibitor of p53 (references 22–24). Isoform-specific semiquantitative RT-PCR assay from total RNA extracted from human tissues (see Table I). Two tumor pairs (nos. 21 and 22) had readily detectable ΔNp73 levels in tumor tissues but failed to give detectable ΔNp73 levels in their corresponding normal tissues. Therefore, fold up-regulation could not be quantitated in those two cases. Expression levels, standardized for their corresponding GAPDH values, were used to calculate fold induction as described in Materials and Methods. (B) Up-regulation of ΔNp73 transcripts in a series of unmatched 52 human breast cancers compared with 8 unrelated normal breast tissues. ΔNp73-specific semiquantitative RT-PCR assay from total RNA extracted from tissues. Expression levels were standardized using the corresponding GAPDH value of each sample. The relative expression of ΔNp73 in breast cancers and normal breast tissues is shown. The average normal tissue expression (gray line) is indicated. The arrow marks an arbitrary cut-off, delineating tumors with fivefold or higher ΔNp73 overexpression. 16 of 52 breast cancers (31%) overexpress ΔNp73 levels that were between 6- and 44-fold higher than that of average normal breast tissue. (C) Characterization of the polyclonal anti-ΔNp73 does not recognize TAp73α, TAp73β, or p53. H1299 cells were transfected with empty vector or the indicated expression plasmids and lysates (50 μg protein for lanes 1–5 and 10 μg for lanes 6–9) were immunoblotted with either a cocktail of ER15, GC15, and DO-1 (lanes 6–9) or anti-ΔNp73 (lanes 1–5). (D) Tumor-specific up-regulation of ΔNp73α protein. Cases 9, 14, and 26 (top from left to right) and cases 10, 31, and 1 (bottom) from Table I are shown. Immunoprecipitations of equal amounts of total protein (2 mg each) from matched pairs of homogenized tumor/normal tissues with 1 μg anti-p73 antibody ER15 followed by immunoblotting with polyclonal anti-ΔNp73. Hc, heavy chain. The last lane (top) is a positive control of H1299 lysate transfected with a ΔNp73α expression vector.

mors with deregulated ΔNp73 and/or Ex2Del p73 also tended to show up-regulation of TAp73, the magnitude of the ΔNp73 and/or Ex2Del p73 up-regulation was much higher than that of TAp73 in the majority of cases. Among the 31 tumor pairs with up-regulation of any one or all of these three p73 transcripts, 22 tumors (71%) exhibited preferential up-regulation of ΔNp73 (19 tumors; Table I, left side of fifth column) or Ex2Del p73 (3 tumors; Table I, right side of fifth column). These included 13 ovarian cancers and 2 large benign ovarian tumors, 5 endometrial cancers, 2 breast cancers, 2 cervical cancers, and 2 vulvar cancers. Moreover, 14 of these 22 tumors (67%) exhibited exclusive up-regulation of ΔNp73 and/or Ex2Del p73. ("[redacted]" up-regulation means that the up-regulation of ΔNp73 and/or Ex2Del p73 was more marked than the up-regulation of TAp73). In one case, the tumor showed preferential ΔNp73 up-regulation of >2,700-fold compared with TAp73 expression (tumor no. 4). Only nine tumors

(29%) exhibited an inverse ratio with a disproportional rise of TAp73 compared with their rise in ΔNp73. Thus, taken together, in our series of 31 matched tumors with some form of tumor-specific deregulation of the TP73 gene, 22 tumors (71%) exhibit either exclusive or preferential up-regulation of dominant-negative p73 isoforms.

In our paired tumor series, we had an overall p53 mutational rate of 38%, as determined either by direct sequencing of the PCR-amplified DNA binding domain or by immunocytochemically shown nuclear overexpression (Table I). This prevalence is in agreement with the reported rates of p53 mutations in these tumor types (~40%; reference 25). We reasoned that if ΔNp73 and Ex2Del p73 were indeed p53- and TAp73-directed inhibitors and would act as oncogenes, one would expect to see their expression preferentially in wild-type p53 tumors. Of the 22 tumors with preferential up-regulation of ΔNp73 and/or Ex2Del p73, 21 tumors were available for p53 mutational analysis. Of these 21 tumors, 15 tumors harbored wild-type p53 (71%). In contrast, among the nine cases with preferential up-regulation of TAp73, six tumors harbored mutant p53 (66%). We then analyzed this set of 30 tumors for a correlation between overexpression of dominant-negative forms of p73 and concomitant wild-type p53 status. When all tumors were included, a statistical trend but no significance was found by Student's *t* test. When the unusual tumor number 4 is excluded, which is characterized by a singularly high level of ΔNp73 up-regulation of almost 3,000-fold compared with TAp73 up-regulation plus a mutant p53 status, statistical significance was found ($P = 0.014$). Taken together, a correlation between tumor-specific up-regulation of ΔNp73 or Ex2Del p73 and wild-type p53 status of the tumor cannot be made with this limited set of tumors, although a trend is present. More tumor samples will need to be analyzed in the future to support the hypothesis that the expression of dominant-negative p73 isoforms alleviates the selection pressure for p53 mutations in tumors.

To further investigate whether tumors up-regulate ΔNp73, we determined ΔNp73 transcript levels in a series of 52 unmatched breast cancers and compared them to 8 available normal breast tissues from unrelated individuals (Fig. 2 B). 16 of 52 breast cancers (31%) overexpressed ΔNp73 levels that were between 6- and 44-fold higher than the average of the 8 normal breast tissues (Fig. 2 B, grey line). An additional 10 tumors showed ΔNp73 up-regulation between two- and sixfold above the normal average. In contrast, four normal breast tissues showed non-detectable levels of ΔNp73, two cases expressed at average level, and only two tissues were elevated two- to fourfold. Next, we used isoform-specific semiquantitative RT-PCR to simultaneously measure ΔNp73 and TAp73 because we previously showed that breast cancers can also overexpress TAp73 (17). Among the 16 cancers with a 6–44-fold increase of ΔNp73, 12 cancers again showed preferential up-regulation of ΔNp73 over TAp73 (unpublished data). Although the data is not complete enough to make strong conclusions, as we had already seen in the gynecological cancers on Table I, our results on breast cancer again suggests that ΔNp73 might selectively be up-regulated during tumorigenesis.

To confirm that tumor-specific up-regulation of ΔNp73 transcripts translate to the up-regulation of proteins, we generated a ΔNp73-specific polyclonal antibody raised against the unique exon 3′. This antibody recognizes ΔNp73 but does not cross react with TAp73α, TAp73β, or p53 (Fig. 2 C). Using this reagent, we determined ΔNp73 protein expression on 10 matched pairs of homogenized tumor/normal tissues from Table I. Tissues were subjected to immunoprecipitations of equal amounts of total protein (2 mg each) with the anti-p73 specific antibody ER15 followed by immunoblotting with polyclonal anti-ΔNp73. Some examples are shown in Fig. 2 D, which represent cases 1, 9, 10, 14, 26, and 31. Tumor-specific up-regulation of ΔNp73 protein was found in all 10 cases as demonstrated by tumors yielding detectable ΔNp73α protein, whereas their respective matched normal tissue showed a complete absence of ΔNp73α protein in nine cases and only a minute amount in case number 10. Moreover, when tumor lysates in these cases (2 mg each) were immunoprecipitated with nonspecific Flag antibody, ΔNp73 protein could not be detected (unpublished data). Also, the immunoprecipitation of cases 9, 14, and 26 with β-specific anti-p73 antibody GC15 did not yield ΔNp73 protein, indicating that in contrast to ΔNp73α, ΔNp73β is not up-regulated to detectable levels in these cases. As expected, no strict correlation between the levels of tumor-associated protein and the ranking in Table I is present because the RT-PCR measurements indicate the relative fold increase of mRNA levels of tumor versus normal rather than absolute values. Interestingly, cases 26 and 31 did not exhibit increased ΔNp73 expression of their tumors at the transcript level, yet clearly do so on the protein level. This notion warrants additional investigation because it might suggest that the prevalence of tumor-associated ΔNp73 up-regulation is somewhat higher than transcript measurements would predict.

ΔNp73 Is an Efficient Dominant-Negative Inhibitor of the Transcription Function of Wild-type p53 and TAp73. To test the hypothesis that human ΔNp73 is a dominant-negative inhibitor of human wild-type p53 and TAp73, we first performed reporter assays with expression plasmids for wild-type p53, TAp73α, TAp73β, and a p53/TAp73-responsive luciferase reporter in the presence or absence of ΔNp73α using p53 null H1299 and SaOs2 cells (Fig. 3, A–C, and unpublished data). Increasing ratios of ΔNp73α expression plasmid were added to a constant amount of p53 or TAp73α and TAp73β plasmids. Fig. 3 B shows that the p53 expression levels in this assay were independent of the amount of ΔNp73α added, and that p53 and TAp73 expression levels were not interfering with the amount of ΔNp73α expressed. Surprisingly, the steady state levels of TAp73β actually increased in proportion to the amount of ΔNp73α added, despite the fact that the same amount of TAp73β plasmid was used in all reactions. Of note, the addition of ΔNp73α exhibited complete suppression of the transcriptional activity of wild-type p53, TAp73α, and

TAp73β in a dose-dependent manner (Fig. 3 A and unpublished data). Furthermore, ΔNp73α also efficiently suppresses endogenous target gene products of wild-type p53 and TAp73 (Fig. 3 C and unpublished data). In HeLa and H1299 cells, the transfection of wild-type p53 or TAp73β induces endogenous HDM2, 14-3-3σ and p21Waf1 compared with basal levels seen with empty vector. However, the concomitant expression of ΔNp73α strongly suppresses each of these response gene products (compare lanes 2 and 3 and lanes 4 and 5). Expression of ΔNp73α alone did not suppress these target genes (compare lanes 6 and 7).

greek sigma symbol

ΔNp73 Is an Efficient Dominant-Negative Inhibitor of the Apoptosis and Suppressor Function of Wild-type p53 and TAp73. Moreover, ΔNp73α is a strong inhibitor of apoptosis induced by wild-type p53 and TAp73 (Fig. 4 A). HeLa and SaOs2 cells undergo wild-type p53- and TAp73-dependent cell death as assessed by Annexin V staining and TUNEL assay. This apoptotic activity is completely abolished by the coexpression of ΔNp73α (Fig. 4 A and unpublished data). The inhibitory action of ΔNp73α is dependent on the presence of transcription-competent wild-type p53 and TAp73 because ΔNp73α alone cannot affect apoptosis (Fig. 4 A, left column). Furthermore, in agreement with the antiapoptotic effect, ΔNp73α is an inhibitor of colony suppression mediated by wild-type p53 and TAp73 (Fig. 4 B and Table II). The reintroduction of wild-type p53 and TAp73 suppresses the growth of SaOs2 cells (2, 26) and this suppression is thought to be largely due to apoptosis (27). In keeping with these results, the transfection of wild-type p53 strongly suppresses macroscopic colony formation of SaOs2 cells compared with many visible colonies with vector backbone alone (4 foci for wild-type p53 vs. 1,778 for vector control; Fig. 4 B and Table II). As expected, the expression of ΔNp73α alone has no growth-promoting effect compared with vector controls (1,383 vs. 1,778 foci), but was actually slightly growth suppressive in these p53 null cells for reasons that are unclear. In contrast, the coexpression of ΔNp73α with

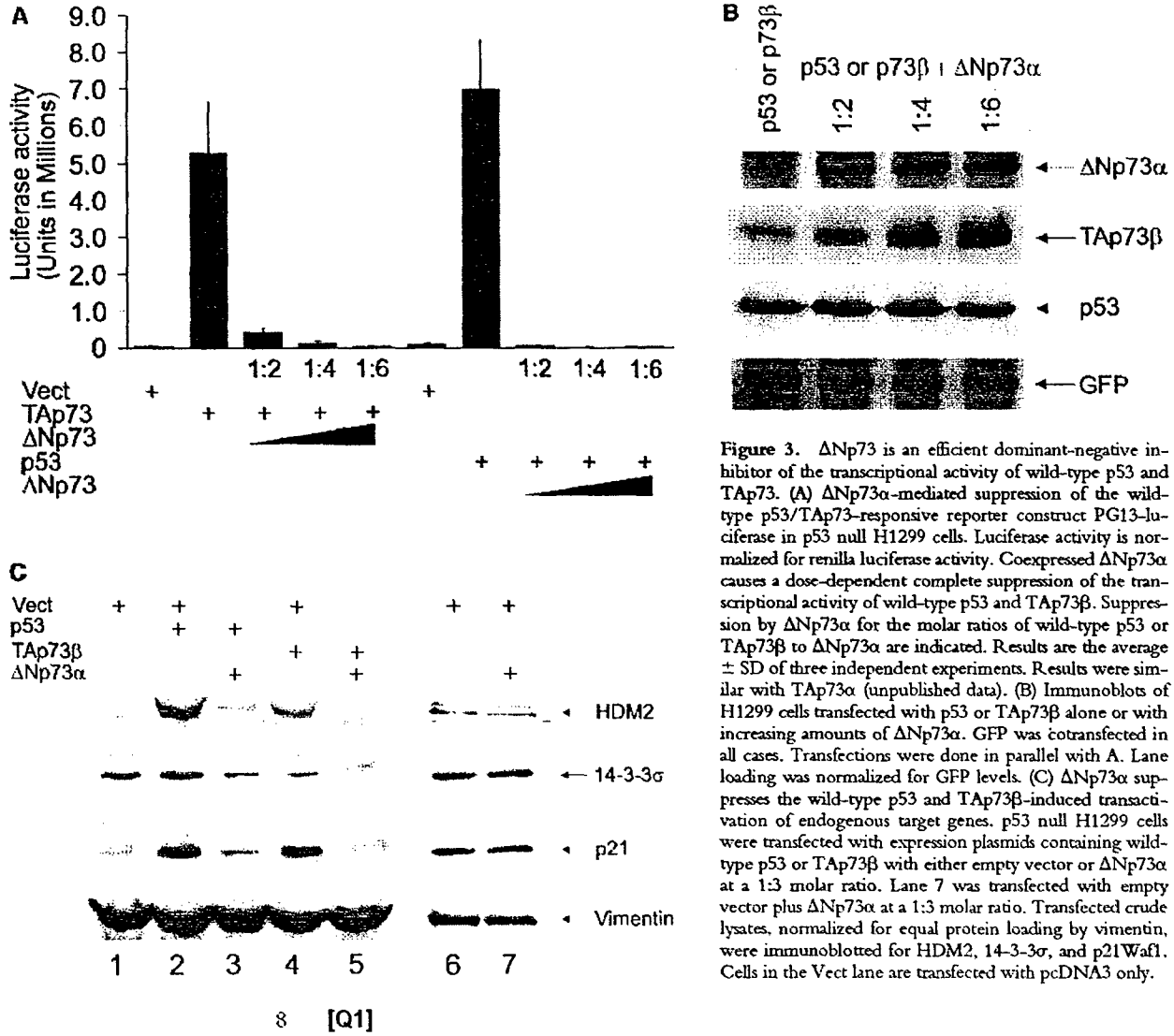


Figure 3. ΔNp73 is an efficient dominant-negative inhibitor of the transcriptional activity of wild-type p53 and TAp73. (A) ΔNp73α-mediated suppression of the wild-type p53/TAp73-responsive reporter construct PG13-luciferase in p53 null H1299 cells. Luciferase activity is normalized for renilla luciferase activity. Coexpressed ΔNp73α causes a dose-dependent complete suppression of the transcriptional activity of wild-type p53 and TAp73β. Suppression by ΔNp73α for the molar ratios of wild-type p53 or TAp73β to ΔNp73α are indicated. Results are the average ± SD of three independent experiments. Results were similar with TAp73α (unpublished data). (B) Immunoblots of H1299 cells transfected with p53 or TAp73β alone or with increasing amounts of ΔNp73α. GFP was cotransfected in all cases. Transfections were done in parallel with A. Lane loading was normalized for GFP levels. (C) ΔNp73α suppresses the wild-type p53 and TAp73β-induced transactivation of endogenous target genes. p53 null H1299 cells were transfected with expression plasmids containing wild-type p53 or TAp73β with either empty vector or ΔNp73α at a 1:3 molar ratio. Lane 7 was transfected with empty vector plus ΔNp73α at a 1:3 molar ratio. Transfected crude lysates, normalized for equal protein loading by vimentin, were immunoblotted for HDM2, 14-3-3σ, and p21Waf1. Cells in the Vect lane are transfected with pcDNA3 only.

wild-type p53 at a 1:1 molar ratio counteracts this effect, leading to a 12.5-fold increase in the number of colonies from 4 to 51 foci. Likewise, TAp73α, although not quite as potent as wild-type p53, suppresses colony formation (82 foci), but the coexpression of ΔNp73α with TAp73α again antagonizes this effect and increases the number of macro-

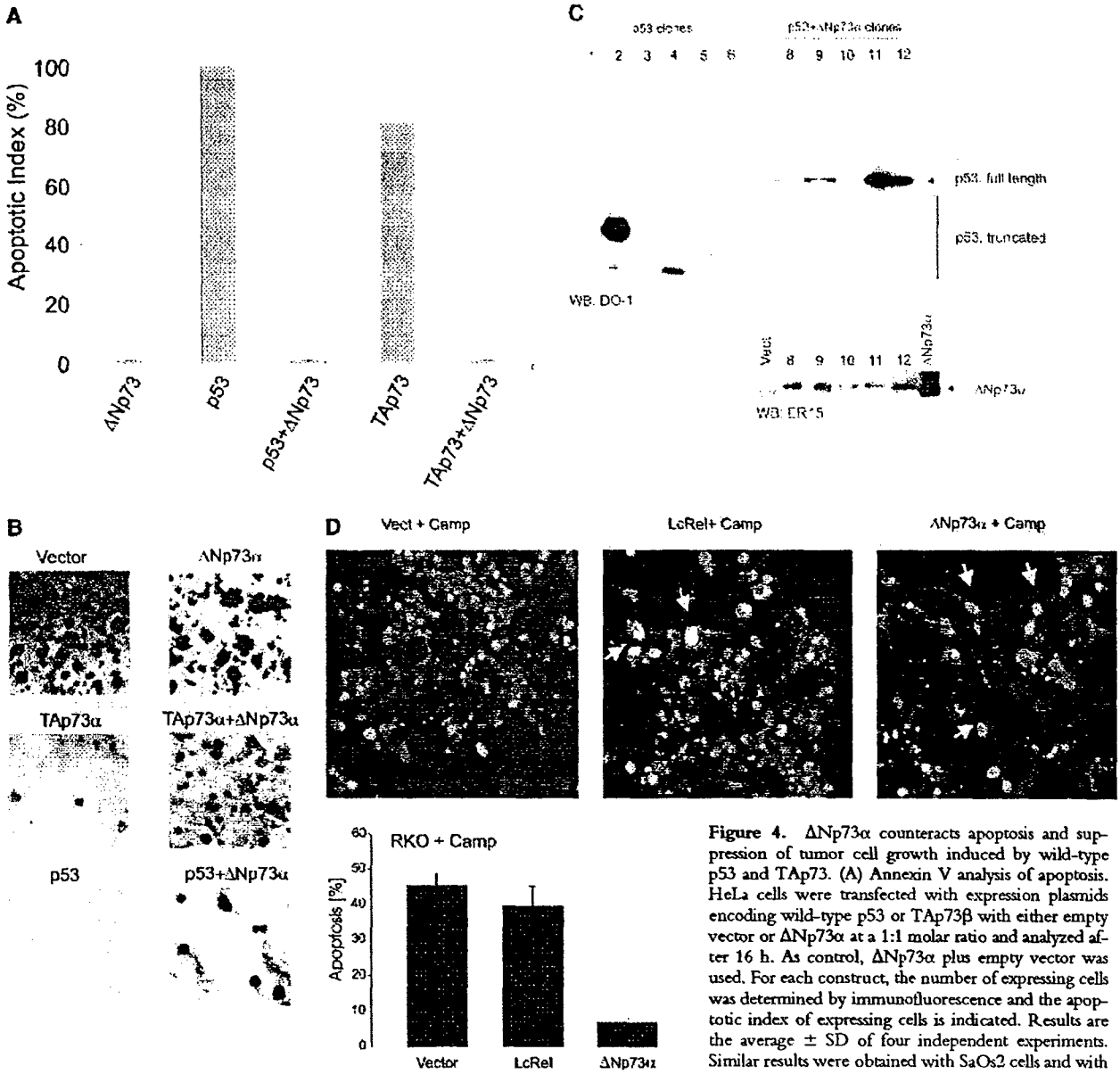


Figure 4. ΔNp73α counteracts apoptosis and suppression of tumor cell growth induced by wild-type p53 and TAp73. (A) Annexin V analysis of apoptosis. HeLa cells were transfected with expression plasmids encoding wild-type p53 or TAp73β with either empty vector or ΔNp73α at a 1:1 molar ratio and analyzed after 16 h. As control, ΔNp73α plus empty vector was used. For each construct, the number of expressing cells was determined by immunofluorescence and the apoptotic index of expressing cells is indicated. Results are the average ± SD of four independent experiments. Similar results were obtained with SaOs2 cells and with TUNEL analysis. (B) SaOs2 colony suppression induced by wild-type p53 and TAp73α is inhibited by ΔNp73α. Number of colonies are shown in Table II. (C) p53 expression analysis of random SaOs2 clones derived from surviving colonies of a parallel experiment to the one shown in B. Immunoblots with anti-p53 antibody DO-1 using 30 μg total cell lysate per lane. Clones from cells originally transfected with expression plasmids for wild-type p53 alone (left) or wild-type p53 plus ΔNp73α (right). Except for clone 6, all other wild-type p53-transfected clones have lost full-length p53 protein expression. Clones 1, 3, and 5 show no detectable p53 protein at all, whereas clones 2, 4, and 6 express truncated p53 polypeptides (left). In contrast, all six colonies derived from cotransfection with wild-type p53 and ΔNp73α show detectable levels of full-length p53 protein with sequence-confirmed wild-type status of the DNA binding domain in three of three tested clones (right, five clones are shown). Immunoblot to show ΔNp73α expression in SaOs2 clones 8–12 compared with a SaOs2 clone transfected with empty vector (bottom). (D) ΔNp73α confers drug resistance to wild-type p53/TAp73 harboring tumor cells. RKO cells were transfected with empty vector (left), the irrelevant expression plasmid LcRel (center), or with ΔNp73α expression plasmid (right). 5 h after transfection, cells were treated with 5 μM camptothecin overnight or left untreated (not depicted). After fixation with paraformaldehyde, each well underwent both TUNEL staining in green and immunofluorescence with ΔNp73-specific polyclonal antibody (left and right) or Flag antibody (center) in red to assess apoptosis and expression. In contrast to vector-only or LcRel-transfected cells, ΔNp73α-expressing cells (white arrows) are virtually protected from camptothecin-induced apoptosis. The p73 antibody produces a slight background staining in empty vector–transfected RKO cells, which allows the counting of all cells in the well (left). The percentage of apoptosis of ΔNp73α-expressing and control-transfected cells after 24 h of treatment with 5 μM camptothecin is shown.

(white arrows, right panel)

scopic colonies by 8.1-fold to 669 foci. Taken together, ΔNp73 is an efficient dominant-negative inhibitor of wild-type p53 and TAp73, although the strong repressive effect of ΔNp73α on p53 and TAp73 in transient assays (Fig. 4 A) is more modest in long-term assays (Fig. 4 B). Entirely consistent with this finding were data from a subsequent p53 expression analysis of SaOs2 cell clones that were established from surviving colonies of a duplicate experiment. A complete loss of full-length p53 protein expression was found in five out of six such randomly picked clones that were derived from plates transfected with wild-type p53 alone (Fig. 4 C, left). Although clones 1, 3, and 5 showed no detectable p53 protein at all, clones 2, 4, and 6 expressed only truncated and presumably nonfunctional p53 polypeptides, likely due to chromosomal rearrangement. The functional significance of the very small amounts of full-length p53 protein detectable in clone 6 is unclear, in light of the presence of coexpressed truncated p53. On the whole, this data is in agreement with the fact that wild-type p53 expression is incompatible with the outgrowth of clones in this assay, and rare colonies escape because they have lost wild-type p53 expression (28). In contrast, all six randomly picked colonies derived from plates cotransfected with wild-type p53 and ΔNp73α exhibited only full-length p53 protein (Fig. 4 C, five clones are shown). Upon sequencing the DNA binding domain of three of these clones, all three revealed wild-type p53 genotypes. All clones expressed elevated levels of ΔNp73α protein compared with endogenous levels (Fig. 4 C). Taken together, this data indicates that the coexpression of ΔNp73α neutralizes the growth-suppressive effect of wild-type p53, thereby removing the selection pressure for the deletion or rearrangement of wild-type p53. ΔNp73α is able to effectively counteract p53- and TAp73-induced colony suppression in transformed human cells.

ΔNp73α Confers Drug Resistance to Wild-type p53-harboring Tumor Cells. Because we have shown that ΔNp73 is an efficient inhibitor of exogenous wild-type p53-mediated apoptosis and growth suppression, we next tested whether ΔNp73α also inhibits endogenous wild-type p53-mediated apoptosis after DNA damage. RKO cells, a human colon cancer line harboring wild-type p53 and TAp73, were transfected with empty vector, an irrelevant control plasmid (LcRel), or with ΔNp73α expression plasmid and treated with either 5 μM camptothecin or left untreated (Fig. 4 D). Camptothecin is a topoisomerase inhibitor generating single and double strand DNA breaks, which induce an increase of p53 and TAp73 protein levels (29). As expected, control cells treated with camptothecin and transfected with either empty vector or LcRel underwent marked apoptosis with 44 and 39% of cells dying after 24 h. In contrast, ΔNp73α-expressing camptothecin-treated cells were strongly protected from drug-induced apoptosis, causing only 6% of cells to die. Thus, ΔNp73α protects human cancer cells from p53- and TAp73-induced cell death mediated by a chemotherapeutic agent.

Table II. *Inhibition of Colony Suppression by ΔNp73*

Plasmid	No. of foci	SD[a]
vect	1,778	213
ΔNp73	1,383	194
wtp53	4	8
wtp53 plus ΔNp73	51	14
TAp73α	82	16
TAp73α plus ΔNp73	669	187

wtp53, wild-type p53.
[a]Data are derived from three independent experiments.

ΔNp73 Inhibits Wild-type p53 and TAp73 Function by Heterocomplex Formation. One explanation for the observed dominant-negative effect is a direct physical interaction between ΔNp73 and either wild-type p53 or TAp73 proteins, analogous to the dominant-negative mode of action of mutant p53 proteins toward wild-type p53. To test this hypothesis directly, lysates prepared from p53 null SaOs2 cells cotransfected with wild-type p53 and ΔNp73α were immunoprecipitated with monoclonal antibody ER15, which recognizes ΔNp73α. Immunoblot analysis with an antibody specific for p53 (CM1) revealed a complex of the two proteins (Fig. 5 A, lane 3). Of note, TAp73 isoforms are unable to form a protein complex with wild-type p53 (Fig. 5 A, lane 7, B, lane 3, and C, lane 1; references 18 and 30–32), excluding the possibility that the observed p53 band was coimmunoprecipitated via the endogenous TAp73 protein of SaOs2 cells. As control, no such complex was seen in SaOs2 cells transfected with either empty vector, TAp73α, or ΔNp73α alone (Fig. 5 A, lanes 1, 2, and 4), nor was a complex seen in SaOs2 cells transfected with p53 alone or with p53 plus TAp73α (Fig. 5 A, lanes 6 and 7), indicating the specificity of the p53–ΔNp73α complex. Moreover, a similar complex was seen in wild-type p53-expressing human U2OS cells after transfection with ΔNp73α alone. Fig. 5 B shows a specific complex between endogenous wild-type p53 and ectopic ΔNp73α that was immunoprecipitated by ER15 from U2OS cells (lane 1). No such complex was seen when an irrelevant monoclonal antibody against GFP was used (lane 2), or when TAp73α or empty vector were expressed (lanes 3 and 4). The same specific complex can again be immunoprecipitated from U2OS cells using a monoclonal antibody specific for p53 (421) and immunoblotted with the polyclonal antibody specific for ΔNp73 that does not cross react with any TAp73 proteins (Fig. 5 C, lane 2). Again, no such complex is found with preimmune mouse IgG (Fig. 5 C, lane 3) or when U2Os cells were transfected with TAp73α (lane 1) or vector (unpublished data). The lysate lane in Fig. 5 A represents 5% of the IP [Q8] input and the lysate lane in Fig. 5 C represents 2–10% of the IP [Q8] input, depending on the experiment. Together, this data indicates that a small but highly reproducible portion of ΔNp73 forms a stable, specific complex with wild-type p53 in cells. To confirm the existence of this complex in

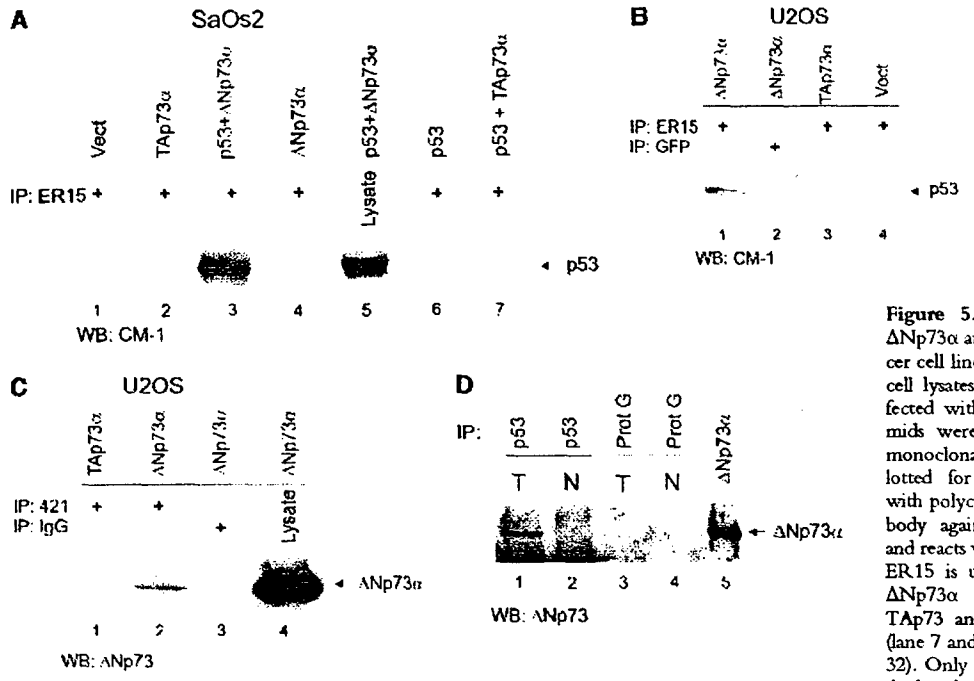


Figure 5. Physical interaction between ΔNp73α and wild-type p53 proteins in cancer cell lines and human tumors. (A) Crude cell lysates of p53 null SaOs2 cells transfected with the indicated expression plasmids were immunoprecipitated with the monoclonal antibody ER15 and immunoblotted for coprecipitating wild-type p53 with polyclonal CM-1. ER15 is a p73 antibody against a COOH-terminal epitope and reacts with both ΔNp73α and TAp73α. ER15 is used here to immunoprecipitate ΔNp73α because complexes between TAp73 and wild-type p53 do not occur (lane 7 and B and C; references 18 and 30–32). Only lysate was loaded in lane 5. The shadow band in lanes 4, 6, and 7 is derived from the heavy chain of added ER15. (B) Crude cell lysates of wild-type p53 harboring U2OS cells transfected with the indicated plasmids were immunoprecipitated with ER15 or an irrelevant monoclonal antibody against GFP and immunoblotted for coprecipitating endogenous p53 with polyclonal CM-1. (C) Crude cell lysates of U2OS cells transfected with the indicated plasmids were immunoprecipitated with a monoclonal antibody against p53 (421) or irrelevant mouse IgG and immunoblotted for coprecipitating ΔNp73α with polyclonal anti-ΔNp73. This antibody is raised and immunopurified against exon 3′ and does not cross react with TAp73 isoforms or p53. Only lysate was loaded in the indicated lane. (D) Detection of tumor-specific protein complexes between ΔNp73α and wild-type p53 in an ovarian carcinoma. Immunoprecipitations of equal amounts of total protein (2 mg each) from a matched pair of homogenized tumor/normal tissues using a mixture of anti-p53 antibodies DO-1 and 1801 covalently coupled to agarose beads, followed by immunoblotting with polyclonal anti-ΔNp73. The ΔNp73α–p53 protein complex is detectable in the tumor but not in the respective normal tissue. Equal amounts of the same tumor and normal tissue lysates immunoprecipitated with protein G–coupled beads are used as an additional negative control. The last lane is a positive control of H1299 lysate transfected with a ΔNp73α expression vector.

vivo in tumor tissues, we coimmunoprecipitated the ΔNp73–p53 complex from an ovarian carcinoma and an endometrial carcinoma with wild-type p53 status (see example in Fig. 5 D, lane 1). Of note, this mixed complex was only seen in the patients' tumors but not in the corresponding normal ovarian tissues (Fig. 5 D, lane 2). Moreover, no specific complex was seen when the immunoprecipitating anti-p53 antibody beads were substituted by protein G beads (lanes 3 and 4).

We also examined the second possibility, namely direct competition for specific DNA binding between ΔNp73α and wild-type p53 at the promoter level. Gel shift assays were performed with two different p53-specific DNA binding sequences as probes, using nuclear extracts of H1299 or U2OS cells transfected with expression plasmids for p53 alone or in combination with ΔNp73α. Although nuclear extract transfected with empty vector showed no specific DNA complex (Fig. 6 A, lane 1), p53 formed a specific complex (lane 2) that could be supershifted with the p53-specific antibody PAb 421 (lane 3) and competed off with 50-fold excess of unlabeled specific probe (lane 4). Likewise, p53 only binds to the specific probe (Fig. 6 A, lane 9), but not to a scrambled version of the specific probe (lane 11). The specific p53/DNA band (Fig. 6 A, lane 9) is supershifted by antibody 421 (lane 10), but 421 cannot produce a "false" supershifted band when p53 fails to bind to the scrambled probe (lane 12). Extracts from cells transfected with ΔNp73α alone failed to bind to both probes despite high levels of ΔNp73α expression on immunoblots (Fig. 6 A, lane 8, and unpublished data). However, nuclear extracts from cells cotransfected with p53 and ΔNp73α at a 1: 4 ratio showed a slight decrease in p53–DNA complex formation (Fig. 6 A, compare lanes 2 and 5), which became more pronounced at a 1:20 ratio of p53 to ΔNp73α (compare lanes 6 and 7). Moreover, in a p53 reporter assay using PG13, a tetramerization-incompetent but DNA binding–competent mutant of ΔNp73α, called ΔNp73 L322P (corresponding to L371P in TAp73α; reference 4), showed a significant but incomplete reversal of the dominant-negative inhibition of p53 transactivation by ΔNp73α (Fig. 6 B). These results support the notion that competition at the promoter site is another mode of inhibition. Taken together, the formation of inactive heterooligomers between p53 and ΔNp73 appear to play a role in the dominant-negative inhibition of p53 by ΔNp73. Such complexes occur naturally in primary tumors and in cultured cells. On the other hand, competition between ΔNp73 and p53 at the level of the promoter, where the presence of ΔNp73 inter-

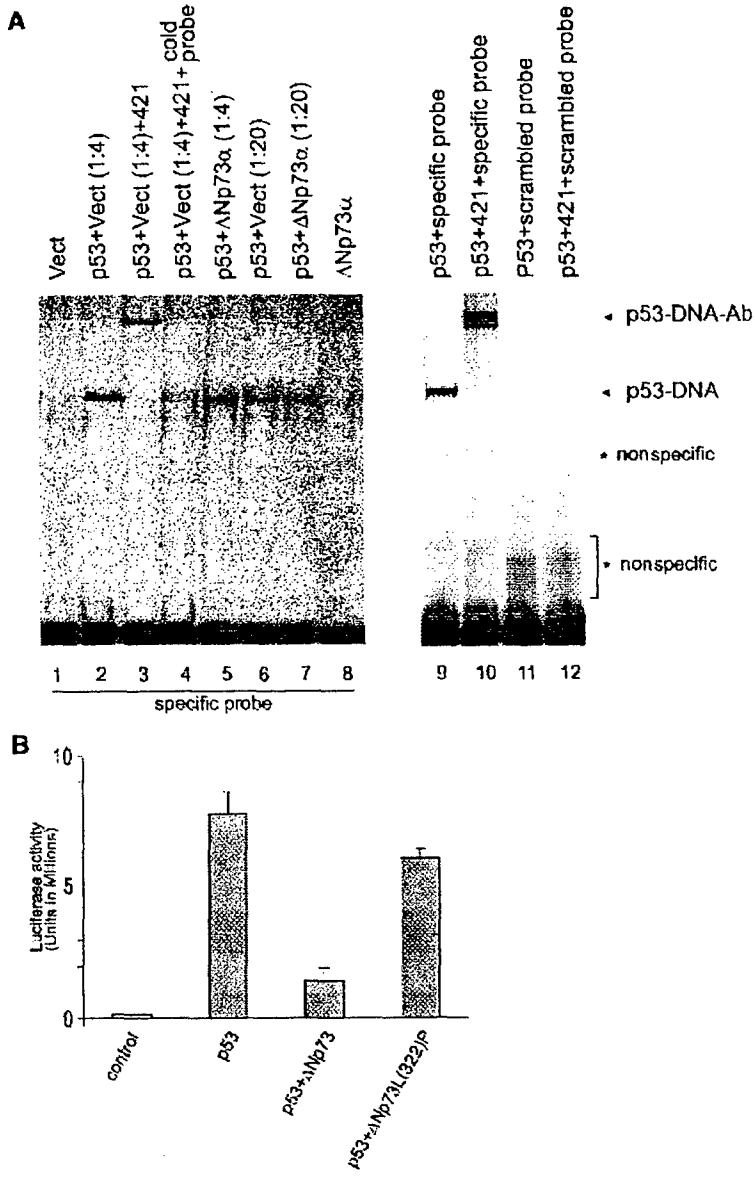


Figure 6. Mechanism of the dominant-negative effect of ΔNp73. (A) EMSA using nuclear extracts of H1299 cells transfected with expression plasmids for p53 or ΔNp73α alone or in combination. Competition of sequence-specific binding of p53 by ΔNp73α is shown (compare lanes 6 and 7). ΔNp73α alone does not bind to the p53 cognate site, p53CON (lane 8). Anti-p53 antibody (421) was added as indicated. (B) p53 reporter assay using the p53-responsive reporter sequence PG13. A tetramerization-incompetent but DNA binding–competent mutant of ΔNp73α, ΔNp73 L(322)P (corresponding to L371P in TAp73α; reference 4), shows a significant but incomplete reversal of the dominant-negative inhibition of p53 transactivation by ΔNp73α.

feres with the ability of p53 to bind effectively to its cognate binding site, might be a second mechanism that is physiologically relevant. Using nuclear extracts of ΔNp73-transfected cells, however, we failed to obtain clear evidence that ΔNp73α alone can directly bind to p53 cognate sites. This issue justifies additional studies.

Suppression of ΔNp73 Enhances Apoptosis Mediated by p53 and TAp73. Our studies demonstrate a dominant-negative action of ΔNp73 toward the apoptosis, focus suppression, and transactivation functions of p53 and TAp73 within the context of forced ΔNp73 expression. To test whether ΔNp73 would have the same inhibitory effect in cells when present at endogenous levels, we used antisense oligonucleotides to suppress endogenous ΔNp73 expression in wild-type p53-harboring RKO cells that undergo apoptosis after DNA damage. RKO cells treated with camptothecin alone underwent a certain level of apoptosis (Fig. 7 A, left bar). In contrast, the preincubation of RKO cells with antisense oligonucleotides directed against the unique exon 3′ of ΔNp73 showed a significant enhancement of p53-mediated apoptosis after camptothecin stress (Fig. 7 A, center bar), whereas camptothecin-stressed RKO cells pretreated with the sense version of the same oligonucleotide did not (Fig. 7 A, right bar). To further confirm that the gain in apoptotic ability seen after down-regulation of endogenous ΔNp73 was in fact due to specific derepression of p53-mediated apoptosis, we modified the above experiment by directly transfecting p53. RKO cells were transfected with wild-type p53 expression plasmid before incubation with antisense or sense oligonucleotides. As al-

ready seen in Fig. 7 A, p53-expressing cells treated with antisense oligonucleotides showed significantly enhanced apoptosis compared with the same cells treated with sense oligonucleotides (Fig. 7 B). To confirm that our antisense strategy works, we determined ΔNp73 protein levels. As shown in Fig. 7 C, transfection of the antisense oligonucleotide clearly down-regulated endogenous ΔNp73 protein levels compared with the sense control oligonucleotide. Taken together, these studies clearly indicate that endogenous ΔNp73 exerts a significant transdominant inhibition of p53 function. Down-regulation of endogenous ΔNp73 levels alleviates its suppressive action on p53-mediated apoptosis after DNA damage.

Discussion

p53 controls a powerful stress response by integrating signals from many types of DNA damage or inappropriate oncogenic stimulation. Activation of p53 elicits a cellular response of apoptosis, cell cycle arrest, or senescence, thereby preventing tumor formation. Therefore, direct or indirect loss of p53 function is a preeminent defect in most human cancers whether via intragenic mutation of p53 itself (33), lack of p53 nuclear retention (34), loss of its upstream activator p14ARF (35), or inhibition by its antagonist HDM2 (36, 37). Here we show that the human TP73 gene, a homologue of p53, produces an NH_2 terminally truncated isoform driven by an alternative internal promoter in intron 3. Human ΔNp73 starts with a unique exon 3′ that is highly conserved from mouse exon 3′ and is in frame with exon 4. ΔNp73 lacks the transactivation domain and is therefore predicted to function as a transdominant inhibitor of p53. In physical and functional assays, we demonstrate dominant-negative interactions between human ΔNp73 and wild-type p53 or transcription-competent TAp73. ΔNp73 is a potent inhibitor of wild-type p53 and TAp73 with respect to their transcriptional activation, apoptotic ability, and growth suppressor function. Of note, ΔNp73-mediated interference with the biological p53 response occurs at endogenous ΔNp73 levels, because antisense-directed down-regulation of endogenous ΔNp73 expression leads to a derepression of the p53 response.

Importantly, in this study we provide the first clinical evidence that ΔNp73 is frequently up-regulated in a variety of primary cancers including cancers of the breast and the female genital tract. We show that tumor-specific up-regulation of ΔNp73 occurs at the mRNA and protein level in primary tumors. In a rigorous comparison of tumor/normal tissue pairs from 37 patients, 73% show ΔNp73 up-regulation specifically in their tumor tissues but not in their respective normal tissues of origin. In addition, a subset of those tumors also up-regulate the previously described Ex2Del p73, another transdominant inhibitor of p53, but one that is generated from the P1 promoter of TP73 via alternative splicing. Taken together, 81% of tumor pairs exhibited tumor-specific up-regulation of ΔNp73 and/or Ex2Del p73. Of note, among the tumors with up-regulation of any one or all three TP73 transcripts (ΔNp73, Ex2Del p73, and TAp73), 71% exhibited preferential up-regulation of ΔNp73 or Ex2Del p73. Moreover, two thirds of the latter group of tumors showed exclusive up-regulation of ΔNp73 and/or Ex2Del p73 without any concomitant rise in TAp73. Furthermore, among tumors with preferential up-regulation of ΔNp73 and Ex2Del p73, 71% harbored wild-type p53. In contrast, among the (small) group of tumors with preferential TAp73 up-regulation,

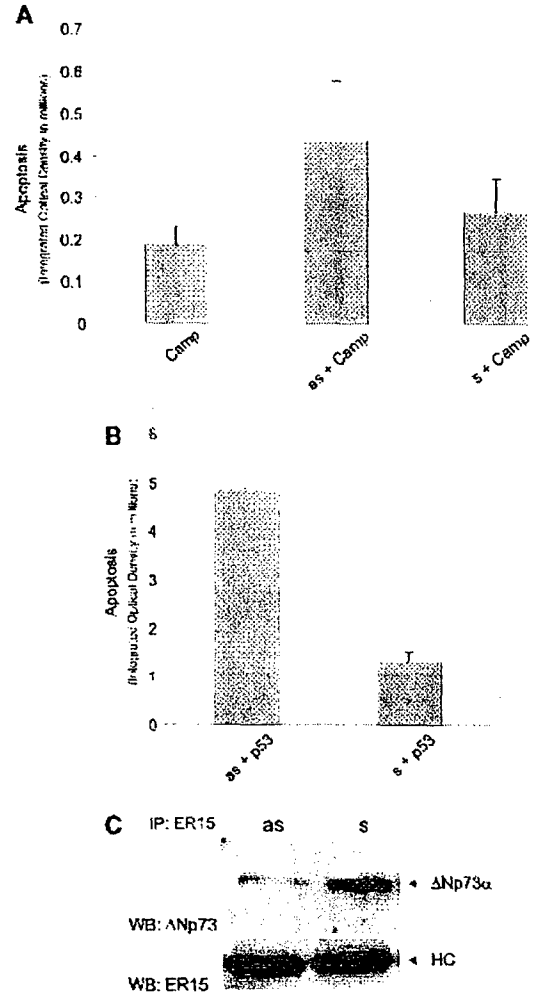


Figure 7. Down-regulation of endogenous ΔNp73 levels alleviates its suppressive action and thereby enhances apoptosis mediated by p53 and TAp73. (A) Wild-type p53 harboring RKO cells were transfected with antisense (as) or sense (s) oligonucleotides (200 nM each) directed against exon 3′ of DNp73. After 8 h, cells were subjected to DNA damage by 1 mM camptothecin for an additional 16 h before TUNEL staining. Apoptosis was determined by measuring green fluorescence as described in Materials and Methods. Camp, cells treated with camptothecin alone. (B) RKO cells were transfected with 360 ng wild-type p53 expression plasmid with 200 nM of either ΔNp73 antisense (as) or sense (s) oligonucleotides. 20 h after transfection, TUNEL staining was performed. Apoptosis was quantitated as described above. (C) Cells were transfected with 200 nM each of antisense (as) or sense (s) oligonucleotides. 36 h later, equal amounts of lysates (2 mg total protein) were immunoprecipitated with ER15 and blotted with the polyclonal anti-ΔNp73.

66% harbored mutant p53. In summary, given the trend between tumor-specific up-regulation of ΔNp73 and/or Ex2Del p73 on the one hand and wild-type p53 status on the other, it is tempting to speculate that the expression of dominant-negative p73 isoforms alleviates the selection pressure for p53 mutations in tumors by functionally inactivating the suppressor action of p53. More tumor samples need to be analyzed to clarify this question. If confirmed, however, this mode of p53 inactivation would be functionally equivalent to the amplification of the HDM2 gene in some human sarcomas and leukemias (36, 37). While this work was in progress, Grob et al. (38) reported that p53 and TAp73 can directly induce ΔNp73 via a p53-responsive element in the ΔNp73 promoter in cultured cells. Moreover, Nakagawa et al. (39) confirmed that TAp73 directly transactivates ΔNp73, but such an activity for p53 could not be found. Nevertheless, these results further fortify the analogy to the p53-HDM2 autoregulatory feedback loop. It will be interesting to determine whether p53 and/or TAp73 itself drive the overexpression of ΔNp73 in primary tumors as well.

We provide evidence that physical interaction between ΔNp73α and wild-type p53 is one of the possible functional mechanisms of this inhibition. We showed that this mixed complex occurs naturally in cultured human tumor cells and tumor tissues. While this work was under review, Nakagawara et al. (39) also reported that ΔNp73α and ΔNp73β form stable mixed complexes with endogenous and ectopic wild-type p53, as well as with TAp73α. Similarly, Pozniak et al. (18) reported a direct physical interaction of mouse ΔNp73α and ΔNp73β proteins with wild-type p53, both of which inhibited the apoptosis-promoting activity of p53. Thus, heterocomplexes appear to interfere with the specific DNA binding activity of wild-type p53. In contrast to ΔNp73, TAp73 isoforms are unable to form a protein complex with wild-type p53 (Fig. 5, B and C; references 18 and 30–32). Therefore, the existence of mixed complexes suggests that the unique exon 3′ at the NH_2 terminus of ΔNp73, in conjunction with the missing transactivation domain, induces a conformational change in ΔNp73 that allows the complex to form. In contrast, another recently described isoform named ΔTA-p73α, which is encoded by p73 (exons 4–14) but missing the unique exon 3′, does not appear to form a complex with wild-type p53 (40) [Q9]. Heterooligomeric complexes mirror the ability of many missense p53 mutants in heterozygous tumors to abrogate the function of the remaining wild-type p53 allele (41, 42). Of note, a stoichiometry as low as 1:3 of mutant to wild-type p53 molecules already abrogates wild-type p53 function, suggesting that a similar stoichiometry might be effective for ΔNp73 as well. An additional mechanism of p53 inhibition might be direct promoter competition, with ΔNp73 displacing p53 from the DNA binding site (43). Such a phenomena has been seen in gel shift assays with high concentrations of in vitro translated p73 (exons 4–14) protein (ΔTA-p73α; reference 40). In nuclear extracts of H1299 and U2OS cells cotransfected with p53 plus ΔNp73, which more closely mimics the physiological situation than in vitro translated proteins, we also observed a decrease in p53–DNA complex formation in the presence of ΔNp73α, supporting the notion of promoter competition. Under these experimental conditions, however, we failed to obtain clear evidence that ΔNp73α alone can directly bind to p53 cognate sites. Although both inhibitory mechanisms are likely, this point needs additional study.

Responsiveness to oncogenes and selected forms of DNA damage might suggest a putative tumor suppressor role of the TP73 gene analogous to TP53. However, tumor-associated overexpression of (total) p73 and in some cases TAp73 rather than loss of expression, is the most commonly observed tumor-specific abnormality of the TP73 gene. This fact, in conjunction with a conspicuous lack of p73 mutation in human tumors and a lack of a cancer phenotype in p73-deficient mice, provides clear evidence that the TP73 gene is not a classic Knudson-type tumor suppressor in vivo. Instead, our finding that a significant percentage of human tumors specifically select for dominant-negative p73 isoforms strongly argues for their oncogenic role during tumorigenesis in vivo. Preferential up-regulation of ΔNp73 and Ex2Del p73 in tumors might bestow oncogenic activity upon the TP73 gene that specifically interferes with the tumor suppressor functions of wild-type p53 and TAp73. In fact, this scenario suggests that the TP73 gene embodies the "two genes in one" idea with products that play opposing roles within the family circuitry. Their impact on tumor formation in vivo might therefore depend on the balance between tumor-promoting and -suppressing family members. The existence of this inhibitory family network might explain the paucity of p73 mutations in human tumors. In the developing mouse brain, ΔNp73 is the predominant form of TP73 and a powerful inhibitor of p53 (18). In vivo and in vitro studies showed that ΔNp73 plays an essential antiapoptotic role required to counteract p53-mediated neuronal death during the "sculpting" of the developing brain. This explains why $p73^{-/-}$ mice, missing all forms of p73 including protective ΔNp73, underwent accelerated neuronal death in sympathetic ganglia (18). Thus, in keeping with a common theme in cancer, a developmental inhibitory network that is essential for normal physiology resurfaces in cancer, but in a corrupted and derailed fashion. In support of the idea that ΔNp73 can act as an oncogene in vivo, overexpression of ΔN isoforms of p63, a p73 homologue, is found in human cancers including lung cancer, squamous cell carcinoma of the head and neck (44), bladder cancer (45), and nasopharyngeal carcinoma (46). Importantly, a specific ΔNp63 isoform is oncogenic in nude mice and in the Rat 1a focus formation assay (47).

Evidence from some human cancers and mouse models indicates that the mutational status of p53 is an important clinical variable for prognosis as well as for guiding the aggressiveness of anticancer therapy. However, currently, p53 mutational status is not widely accepted as a clinical indicator because many studies show that its prognostic power for survival and its predictive value for therapeutic response is poor (for review see reference 47). From a practical stand-

point, the discovery of a ΔNp73-based p53–p73 interference network suggests that the p53 status of a tumor should no longer be considered in isolation. The existence of this inhibitory network might account for the inconsistencies of clinical studies that sought to use p53 status as a predictor of outcome. It mandates that we do a careful analysis of the functional consequences of this network in vivo. The establishment and clarification of an inhibitory p53–p73 network would have a major clinical impact ranging from fine tuning the prognostic power of p53 mutation status to rational p53–p73-targeted drug design.

In conclusion, we report here the cloning of human ΔNp73 and show that ΔNp73 mediates a novel inactivation mechanism of wild-type p53 and TAp73 via dominant-negative interference. Deregulated expression of ΔNp73 can bestow oncogenic activity upon the TP73 gene by functionally inactivating the suppressor action of p53 and TAp73. This trait might be frequently selected for in a variety of human cancers.

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References

1. Kaghad, M., H. Bonnet, A. Yang, L. Creancier, J.C. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lelias, X. Dumont, et al. 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell.* 90:809–819.
2. Jost, C.A., M.C. Marin, and W.G. Kaelin, Jr. 1997. p73 is a simian p53-related protein that can induce apoptosis. *Nature.* 389:191–194.
3. Zhu, J., J. Jiang, W. Zhou, and X. Chen. 1998. The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer Res.* 58:5061–5065.
4. Zaika, A., M. Irwin, C. Sansome, and U.M. Moll. 2001. Oncogenes induce and activate endogenous p73 protein. *J. Biol. Chem.* 276:11310–11316.
5. Irwin, M., M.C. Marin, A.C. Phillips, R.S. Seelan, D.I. Smith, W. Liu, E.R. Flores, K.Y. Tsai, T. Jacks, K.H. Vousden, et al. 2000. Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature.* 407:645–648.
6. Lissy, N.A., P.K. Davis, M. Irwin, W.G. Kaelin, and S.F. Dowdy. 2000. A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. *Nature.* 407:642–645.
7. Stiewe, T., and B.M. Putzer. 2000. Role of the p53-homologue p73 in E2F1-induced apoptosis. *Nat. Genet.* 26:464–469.
8. Gong, J.G., A. Costanzo, H.Q. Yang, G. Melino, W.G. Kaelin, Jr., M. Levrero, and J.Y. Wang. 1999. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. *Nature.* 399:806–809.
9. Yuan, Z.M., H. Shioya, T. Ishiko, X. Sun, J.Gu, Y.Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum, and D. Kufe. 1999. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. *Nature.* 399:814–817.
10. Agami, R., G. Blandino, M. Oren, and Y. Shaul. 1999. Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis. *Nature.* 399:809–813.
11. Costanzo, A., P. Merlo, N. Pediconi, M. Fulco, V. Sartorelli, P.A. Cole, G. Fontemaggi, M. Fanciulli, L. Schiltz, G. Blandino, et al. 2002. DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. *Mol. Cell.* 9:175–186.
12. Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N.J. Dyson. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell.* 85:537–548.
13. Field, S.J., F.Y. Tsai, F. Kuo, A.M. Zubiaga, W.G. Kaelin, Jr., D.M. Livingston, S.H. Orkin, and M.E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell.* 85:549–561.
14. Yang, A., N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, et al. 2000. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature.* 404:99–103.
15. Moll, U.M., S. Erster, and A. Zaika. 2001. p53, p63 and p73 - solos, alliances and feuds among family members. *Biochim. Biophys. Acta.* 1552:47–59.
16. Kovalev, S., N.D. Marchenko, S. Swendeman, M. LaQuaglia, and U.M. Moll. 1998. Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines. *Cell Growth Differ.* 9:897–903.
17. Zaika, A.I., S. Kovalev, N.D. Marchenko, and U.M. Moll. 1999. Overexpression of the wild type p73 gene in breast cancer tissues and cell lines. *Cancer Res.* 59:3257–3263.
18. Pozniak, C.D., S. Radinovic, A. Yang, F. McKeon, D.R. Kaplan, and F.D. Miller. 2000. An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. *Science.* 289:304–306.
19. Marchenko, N.D., A.I. Zaika, and U.M. Moll. 2000. Death signal induced localization of p53 protein to mitochondria: a potential role in apoptotic signaling. *J. Biol. Chem.* 275: 16202–16212.
20. Baker, S.J., S. Markowitz, E.R. Fearon, J.K. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild type p53. *Science.* 249:912–915.
21. Ostermeyer, A.G., E. Runko, B. Winkfield, B. Ahn, and U.M. Moll. 1996. Cytoplasmically sequestered wild-type p53 protein in neuroblastoma is relocated to the nucleus by a C-terminal peptide. *Proc. Natl. Acad. Sci. USA.* 93:15190–15194. **[Q10]** now inserted on page 3, under "EMSA"
22. Ng, S.W., G.K. Yiu, Y. Liu, L.W. Huang, M. Palnati, S.H. Jun, R.S. Berkowitz, and S.C. Mok. 2000. Analysis of p73 in human borderline and invasive ovarian tumor. *Oncogene.* 19: 1885–1890.
23. Fillippovich, I., N. Sorokina, M. Gatei, Y. Haupt, K. Hobson, E. Moallem, K. Spring, M. Mould, M.A. McGuckin, M.F. Lavin, et al. 2002. Transactivation-deficient p73alpha (p73Deltaexon2) inhibits apoptosis and competes with p53. *Oncogene.* 20:514–522.
24. O'Nions, J., L.A. Brooks, A. Sullivan, A. Bell, B. Dunne, M. Rozycka, A. Reddy, J.A. Tidy, D. Evans, P.J. Farrell, et al. 2001. p73 is over-expressed in vulval cancer principally as the Delta2 isoform. *Br. J. Cancer.* 85:1551–1556.
25. IARC p53 Database. http://www.iarc.fr/p53/Index.html.
26. Diller, L., J. Kassel, C.E. Nelson, M.A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S.J. Baker, B. Vogelstein,

et al. 1990. p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* 10:5772–5781.

27. Pietenpol, J.A., T. Tokino, S. Thiagalingam, W.S. el-Deiry, K.W. Kinzler, and B. Vogelstein. 1994. Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl. Acad. Sci. USA.* 91:1998–2002.
28. Finlay, C.A., P.W. Hinds, and A.J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell.* 57:1083–1093.
29. Zhu, J., S. Nozell, J. Wang, J. Jiang, W. Zhou, and X. Chen. 2001. p73 cooperates with DNA damage agents to induce apoptosis in MCF7 cells in a p53-dependent manner. *Oncogene.* 20:4050–4057.
30. Marin, M.C., C.A. Jost, L.A. Brooks, M.S. Irwin, J. O'Nions, J.A Tidy, N. James, J.M. McGregor, C.A. Harwood, I.G. Yulug, et al. 2000. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. *Nature Genet.* 25:47–54.
31. Di Como, C.J., C. Gaiddon, and C. Prives. 1999. p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. *Mol. Cell. Biol.* 19:1438–1449.
32. Gu, J., D. Chen, J. Rosenblum, R.M. Rubin, and Z.M. Yuan. 2000. Identification of a sequence element from p53 that signals for Mdm2-targeted degradation. *Mol. Cell. Biol.* 20:1243–1253.
33. Vogelstein, B., D. Lane, and A.J. Levine. 2000. Surfing the p53 network. *Nature.* 408:307–310.
34. Moll, U.M., M. LaQuaglia, J. Benard, and G. Riou. 1995. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc. Natl. Acad. Sci. USA.* 92:4407–4411.
35. Sherr, C.J. 1998. Tumor surveillance via the ARF-p53 pathway. *Genes Dev.* 12:2984–2991.
36. Oliner, J.D., J.A. Pietenpol, S. Thiagalingam, J. Gyuris, K.W. Kinzler, and B. Vogelstein. 1993. Oncoprotein MDM2 conceals the activation domain of tumor suppressor p53. *Nature.* 362:857–860.
37. Watanabe, T., A. Ichikawa, H. Saito, and T. Hotta. 1996. Overexpression of the MDM2 oncogene in leukemia and lymphoma. *Leuk. Lymphoma.* 21:391–397.
38. Grob, T.J., U. Novak, C. Maisse, D. Barcaroli, A.U. Luthi, F. Pirnia, B. Hugli, H.U. Graber, V. De Laurenzi, M.F. Fey, et al. 2001. Human DeltaNp73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell Death Differ.* 8:1213–1223.
39. Nakagawa, T., M. Takahashi, T. Ozaki, K. Watanabe, S. Todo, H. Mizuguchi, T. Hayakawa, and A. Nakagawara. 2002. Autoinhibitory regulation of p73 by ΔNp73 to modulate cell survival and death through a p73-specific target element within the ΔNp73 promoter. *Mol. Cell. Biol.* 22:2575–2585.
40. Kern, S.E., J.A. Pietenpol, S. Thiagalingam, A. Seymour, K.W. Kinzler, and B. Vogelstein. 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science.* 256:827–830.
41. Unger, T., M.M. Nau, S. Segal, and J.D. Minna. 1992. p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J.* 11:1383–1390.
42. Ishimoto, O., C. Kawahara, K. Enjo, M. Obinata, T. Nukiwa, and S. Ikawa. 2002 Possible oncogenic potential of DeltaNp73: a newly identified isoform of human p73. *Cancer Res.* 62:636–641.
43. Stiewe, T., C.C. Theseling, and B.M. Putzer. 2002. Transactivation-deficient ΔTA-p73 inhibits p53 by direct competition for DNA binding. *J. Biol. Chem.* 277:14177–14185.
44. Hibi, K., B. Trink, M. Patturajan, W.H. Westra, O.L. Caballero, D.E. Hill, E.A. Ratovitski, J. Jen, and D. Sidransky. 2000. AIS is an oncogene amplified in squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA.* 97:5462–5467.
45. Park, B.J., S.J. Lee, J.I. Kim, S.J. Lee, C.H. Lee, S.G. Chang, J.H. Park, and S.G. Chi. 2000. Frequent alteration of p63 expression in human primary bladder carcinomas. *Cancer Res.* 60:3370–3374.
46. Crook, T., J.M. Nicholl, J. Brooks, J. O'Nions, and M.J. Allday. 2000. High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? *Oncogene.* 10:3439–3444.
47. Moll, U.M. 2000. New p53-based strategies for cancer therapy. *In* DNA Alterations in Cancer: Genetic and Epigenetic Changes. M. Ehrlich, editor. Eaton Publishing, Natick, MA. 439–455.

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